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INACTIVATION OF VIRUSES*

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CONTENTS

Part I Biological Inactivation of Viruses

Part II Chemical Inactivation of Viruses

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Part III Physical Inactivation of Viruses

Part IV Problems to be Considered in the Activation of Viruses

Part I Biological Inactivation of Viruses

NEUTRALIZATION OF VIRAL INFECTIVITY: CHARACTERIZATION OF THE VIRUS-ANTIBODY COMPLEX, INCLUDING ASSOCIATION, DISSOCIATION, AND HOST CELL INTERACTION*

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Introduction

Studies of the neutralization of viruses over the past three decades have provided overwhelming evidence in support of the view that direct interaction between virus and antibody is a necessary antecedent to neutralization. However, attempts to characterize the fundamental aspects of the neutralization reaction have not as yet resulted in a unified and generally accepted concept. Specifically, there is lack of agreement regarding the reversibility of the virus-antibody reaction and the mechanism whereby antibody deprives a virus of its infective capacity.

Although it is conceivable that not all virus-antibody host systems are fundamentally alike, it is very likely that many of the discrepant conclusions about neutralization are the result of varied interpretations of the same experimental findings. This situation is, in part, a consequence of the use of complex organisms for the assay system. Ideally, an assay system should measure accurately and reproducibly the instantaneous status of an *in vitro* reaction and should not modify or permit the continuation of the reaction. The intact animal is unsatisfactory in some or all of these respects. This limitation is well illustrated by the results of Tyrrell and Horsfall,[1] who studied the quantitative relationship between virus and antibody, using as host not only different species of animals but also different routes of inoculation in the same species. These authors found, for example, that for the influenza virus-rabbit antiserum reaction, when assayed in the embryonated egg, a tenfold increase in serum resulted in either a fifty thousandfold or a four hundredfold increase in neutralized virus when assayed by the allantoic cavity or the chorioallantoic route, whereas in mice only a fifteenfold or tenfold increase was obtained by the intranasal or intracerebral route.

These enormous variations, although of intrinsic interest, indicate the undesirability of such assay systems for studies of the reaction between virus and antibody. In a subsequent study employing a tissue culture tube-assay method, Clarke and Tyrrell[2] showed that the high ratios of virus neutralized to concentration of serum were reduced to a value approaching one by removing the inoculum after allowing adsorption of unneutralized virus. These results reveal the difficulty in evaluating the data of the *in vitro* interaction between virus and antibody if the antibody is retained by the host and continues to act not only upon the unneutralized virus in the inoculum but also upon newly released virus.

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Of the viral assay methods currently available, the plaque technique introduced by Dulbecco[3] is the most desirable and was used to obtain the results to be described. The system that has been studied consists of poliovirus Type I (Brunhilde), rabbit immune serum, and HeLa monolayer cultures for plaque assay.[4]

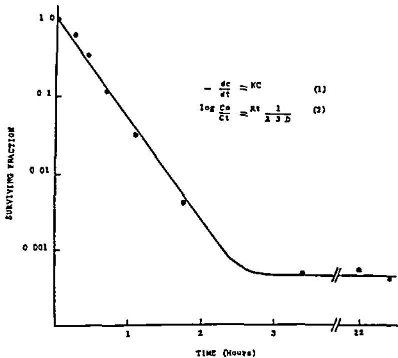


FIGURE 1. The relationship between the amount of unneutralized virus (surviving fraction) and the time of incubation at 37° C. The surviving fraction is plotted on a logarithmic scale against time on an arithmetic scale.

The Association Reaction

Since the neutralization of a virus is the result of its combination with antibody, it is logical to examine first, some of the parameters that affect the association or inactivation reaction. FIGURE 1 illustrates several important features of the reaction. The survival of virus is plotted on a logarithmic scale as a function of time on an arithmetic scale. The curve shows two distinct phases. The first phase is a logarithmic decrease until more than 99.9 per cent of the virus population has been neutralized; the second shows no decrease even over a relatively long period. The latter observation is discussed below. The logarithmic phase can be described by EQUATION 1 (FIGURE 1).

C_a, the concentration at time t for C_t, and the dilution of serum for D The constant K is the reaction rate constant, which is useful as a characteristic of an immune serum, as shown[5] in bacteriophage studies and recently by McBride[6] in studies on the antigenic relationships among poliomyelitis viruses K is also useful as a quantitative index of the effect of a variable on the rate of the virus-antibody reaction

The implication in the exponential decline of virus is that, irrespective of the quantity of virus present, the same fraction will be neutralized per unit of time The same observation was made in 1933 by Andrewes and Elford,[7] who were *studying bacteriophage neutralization* and who described this phenomenon as the "percentage law" namely, the same concentration of antiserum will neutralize the same fraction of virus irrespective (within certain limits) of the original concentration of virus

Another aspect of the reaction of fundamental significance is that inactivation begins immediately The absence of a lag can be interpreted as evidence that a single molecule of antibody is sufficient to cause neutralization of a particle of virus This generalization requires further analysis It is reasonable to assume that there are several adsorption sites on each viral particle A virus that has combined with 1 molecule of antibody has 2 alternative and mutually exclusive ways of reacting with a cell first, adsorption to the cell can occur at a free site, and infection of the cell will ensue, second, adsorption to the cell can occur at the antibody occupied site, but the next step in the infection cycle will be blocked In both instances adsorption to the cell is irreversible and, in the latter instance the virus is considered neutralized

The effect of several variables on the rate of the reaction is shown in FIGURE 2 The solid dots refer to the following conditions $10^{5.4}$ plaque-forming units (pfu) of virus mixed with serum in a final dilution of 1 2000 at a temperature of 37° C The rate constant for this reaction is 117 If the virus input is decreased by a factor of 10, but all other conditions remain unchanged, there is no change in the rate Decreasing the serum by a factor of 2 results in a proportionate decrease in K Finally, decreasing the temperature to 25° C reduces K to 82 These results may be summarized as follows Although the reaction is bimolecular, one of the components (antibody) is present in excess, and the concentration of free virus decreases at a linear exponential rate However, *the rate of the reaction, which is a function of the collision frequency, is* influenced by the concentration of antibody and also by temperature The effect of temperature is to alter the rate constant by a factor of about 1 4 for each 10° C change (that is, Q_{10}), which is indicative of a reaction that is controlled by the diffusion rates of the reactants

The considerations thus far have dealt with the kinetic aspects of the reaction FIGURE 3 shows the quantitative relationships between virus survival and amount of antibody Since the neutralization reaction is dependent both on time and on serum concenentration, the time factor must be controlled in order to study the effect of serum concentration Two curves depicting the quantitative relationship for 2 separate reaction periods are shown The crosses represent the results obtained when the reaction was carried out for 1 hour at 37° C and show, as for the kinetic curve, a linear logarithmic decline

VIRUS	SERUM	TEMP (°C)	K (MIN-1)
—●— 105.3	1 2000	37	117
—+— 104.3	1 2000	37	117
—▲— 105.3	1 4000	37	61
—○— 105.3	1 2000	25	82

SURVIVING FRACTION

1.0
0.1
0.01
0.001

1 2 3 22

TIME (HOURS)

FIGURE 2 The effect of several variables on the rate of neutralization The concentrations of virus and antiserum and the conditions pertaining to each reaction are described in the inset

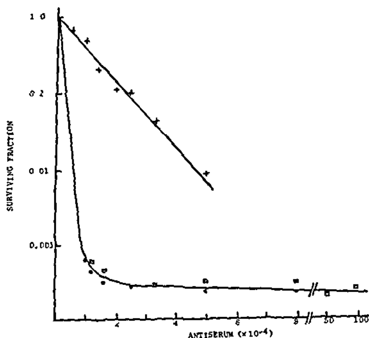


FIGURE 3 Variation in the surviving fraction as a function of the concentration of antiserum In one experiment (+) incubation was for 1 hour at 37° C In 2 experiments (●, □) the virus serum mixtures were incubated for 2 hours at 37° C and for 18 hours at 5° C

with no lag period The second curve, a composite of 2 experiments, describes the status of the same reaction after longer incubation and shows the neutralization of more than 99 9 per cent of the original amount of virus These results reveal, first, that the amount of virus neutralized depends not only on the quantity of antibody present in the reaction mixture but also on the period of time allowed for interaction, and second, that a small fraction (<0 1 per cent) of the total population appears to be resistant to even relatively large concentrations of antibody Studies of this "resistant" fraction are described below However, it can be indicated at present that interaction between this resistant virus and antibody occurs, but without the expected loss in infectivity

The picture one sees of the association reaction between virus and antibody is that of a kinetic process proceeding at a rate governed by the concentration of antibody, by temperature by *p*H (as shown below) and probably by other factors such as the ionic environment On the basis of a constant number of collisions per unit time the exponential decline in surviving virus is to be expected The data just presented are original and are essentially confirmatory of the results of Dulbecco *et al* [8] who reported similar studies of the neutralization of poliovirus and Western equine encephalomyelitis virus (WEE)

The Dissociation Reaction

Previous studies on the reversibility of the virus-antibody reaction as revealed by dilution dissociation have terminated in various conclusions In reviewing the literature Burnet[9] was led to conclude that ' To a near approximation in each case the virus antibody reaction resulting in inactivation is irreversible in the case of typical bacterial viruses and completely reversible in the case of animal viruses " Dulbecco *et al* [8] concluded that for poliovirus there was no evidence for dissociation but that with WEE a slight amount occurred Rubin and Franklin[10] found that neutralized Newcastle disease virus (NDV) underwent between 0 1 and 1 0 per cent dissociation on dilution Kjellen[11] observed dilution reactivation with adenovirus Type 3, but was unable to decide whether this effect was indeed dissociation or only an apparent effect due actually to the decrease in reaction rate as a result of dilution Clarke and Tyrrell[7] could obtain no evidence for dissociation with polio virus but possibly ' some with influenza virus In a critical analysis of the work of Dulbecco and his co-workers Fazekas *et al* [12, 13] have concluded that as a result of operational procedures employed by the former group systematic errors were introduced that if taken into account would make the nondissociation hypothesis untenable

Some of the evidence in favor of the reversibility of the reaction was obtained with intact animal assay systems As mentioned previously the effect of dilution on the reaction rate and the effect of the introduction with retention of antibody are difficult to evaluate and can lead to conclusions of doubtful validity In order to indicate quantitatively how such an apparent effect could arise a theoretical model is shown in TABLE 1 Although this is presented as a model the calcuations are based on experimentally established relationships Let us suppose that a concentration of serum, S, is used that inactivates at a rate k and leaves a surviving fraction of 0 1 per hour If at the time of making

dilutions it is assumed that there are 10,000 survivors, this number will decrease with time, as shown in column 1. If the original mixture is diluted 1:2, 1:5, and 1:10, the number of viral units will be 5000, 2000, and 1000, respectively, and the survivors in each diluted mixture will decrease at a rate in accordance with the dilution factor. It is clear that while, at zero time after dilution, the original mixture contains the highest concentration of virus, after only 2 hours this mixture will contain the least amount and that, after 4 hours, the order of increasing amounts of surviving virus will have completely reversed itself. These results are depicted also in FIGURE 4 and show at a glance the time course that each diluted mixture follows and the extent to which they diverge over a 4-hour period.

Without knowing the concentration of free virus at the time of dilution, the extent of dilution, or the rate of inactivation of the original mixture, the interpretation of results on dilution dissociation becomes precarious.

TABLE 1

THEORETICAL MODEL TO ILLUSTRATE HOW THE RATE OF NEUTRALIZATION CHANGES WHEN A VIRUS ANTISERUM MIXTURE IS DILUTED

	Dilution of the original virus-antiserum mixture			
	1	1:2	1:5	1:10
Serum dilution	S	S/2	S/5	S/10
Surviving fraction C/Co hr $^{-1}$	0.1	0.32	0.64	0.80
Rate constant	K	K/2	K/5	K/10
Hours after dilution	Concentration of virus			
0	10,000	5 000	2,000	1,000
1	1,000	1,600	1,280	800
2	100	500	820	640
3	10	160	550	512
4	1	50	350	410

With these considerations in mind, studies on dilution dissociation were performed.[14] The results of one experiment are shown in TABLE 2. A mixture of antiserum and about 300 pfu of virus was incubated for 2 hours at 37° C and for 18 hours at 5° C, and then an aliquot was diluted fifteenfold. The original and diluted mixtures were assayed after standing for varying periods of time at 37° C, as shown. If the zero-time results are examined, there is only doubtful evidence at best for dilution dissociation. On the other hand, if the 5 hour assays are compared, there would appear to be considerable dissociation involving about 45 per cent of the neutralized virus. It is, of course, obvious that the latter interpretation is erroneous. It is important to ... not react on, which is still pro

2 are therefore spurious and do not reflect a real increase in titer ... remained constant over

In a second experiment a large amount of virus was used and a sufficient quantity of serum added to reduce the titer to the nonneutralizable level. Serial twofold dilutions were then made, and the assays were carried out after

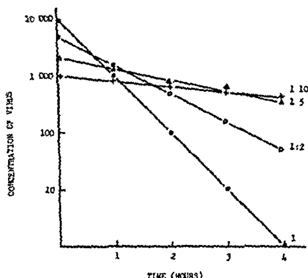


Figure 4. Rate of decrease of active virus in each of the virus antiserum mixtures. Each curve (●—undiluted mixture, ○—1:2, △—1:5, and +—1:10 diluted mixtures) indicates the theoretical concentration of active virus still present at any time during the 4 hour period following the preparation of the respective dilutions.

Table 2

Failure to Obtain Dissociation of Neutralized Virus as a Result of Simple Dilution

Hours at 37° C after dilution	Virus-antiserum mixture*				Viral control			
	Undiluted	1:15 dilution			Undiluted	1:15 dilution		
		Found	Expected	Discrepancy†		Found	Expected	Discrepancy
0	118.3	10.2	7.8	1.31	145.8	10.3	9.7	1.06
2	80.3	10.2	5.3	1.92	—	—	—	—
5	55.3	10.7	3.7	2.90	163.3	13.0	10.8	1.20

* The virus antiserum mixture was prepared with 294 pfu of virus and antiserum in a final dilution of 1:100 000. The mixture was incubated for 2 hours at 37° C and 18 hours at 5° C, at which time dilutions were made.

† The discrepancy is expressed as the ratio of the titer that was found to that expected on the basis of the titer of the undiluted specimen at each of the time intervals shown.

the specimens had been allowed to stand for several hours. Figure 5 shows that some dissociation had occurred. At most, the discrepancy in titer was about 0.7 log, which would correspond to about 0.2 per cent dissociation.

In studies[14] on the effect of pH on the stability of the virus antibody complex, unequivocal evidence for dissociation was obtained as shown in Figure 6.

About 10^5 pfu of virus was mixed with serum and allowed to react for 2 hours at 37° C and 18 hours at 5° C. Aliquots were diluted 1:10 in buffers of various *p*Hs, kept for 2 hours at room temperature, and then assayed. The results show that at *p*H 2.5 virtually all of the neutralized virus had been reactivated. At higher *p*H levels less dissociation occurred. The slight rise shown at *p*H 7 represents dissociation due to dilution. Independent evidence was obtained that antibody activity was not adversely affected by the conditions of the experiment; consequently, the more precise term dissociation is justified rather than reactivation, which may be the result of antibody denaturation.

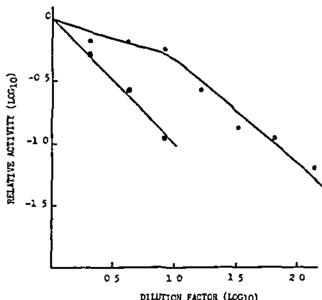


FIGURE 5. The relative decrease in viral activity as a function of dilution. A virus control (●) and a virus serum mixture (O) were diluted serially and assayed for the residual concentration of PFU. The results are plotted as relative values.

The availability of a method for dissociating the virus antibody complex made possible a study[14] of the effect of antibody on the inherent infectiousness of a viral particle. Gard[13] presented evidence that, under prolonged contact of a virus with antibody, the complex underwent a secondary reaction that manifested itself as a loss in dissociability; this process he designated as immunoinactivation. In order to study this phenomenon it is necessary to be able to relate it to 1 of 2 possible events: either to a secondary reaction between virus and antibody *following the primary association reaction* (*this may be considered as a qualitative effect*) or to the result of a continued accretion of antibody (hence a quantitative effect). An experiment was therefore designed to determine whether, in the present system, such "inactivation" occurs and, if so, if it comes under the category of a qualitative or quantitative phenomenon. The results of one experiment are shown in FIGURE 7. About $10^{6.4}$ *pfu of* virus per milliliter and serum (1:4000) were mixed and incubated overnight

On the following day an aliquot of this mixture was diluted one hundredfold in order to reduce to a minimum the further combination between virus and free antibody The 2 virus serum mixtures (that is, undiluted and 1 100 diluted), as well as a viral control were kept at 5° C for as long as 110 days and were assayed periodically for survival and for reactivable (*p*H 2 5) virus

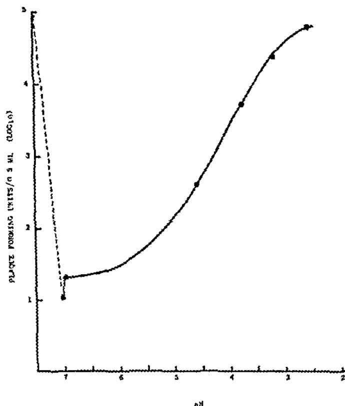


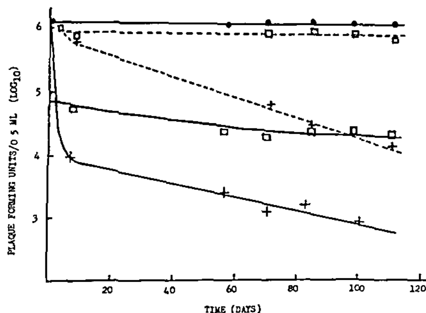
FIGURE 6 Effect of *p*H on the dissociation of the virus antibody complex Virus and antiserum reacted for 2 hours at 37° C and 18 hours at 5° C (*dashed curve*) Aliquots were then adjusted to various *p*Hs and kept for 2 hours at 24° C

The solid curves show the virus survival of the 3 specimens Although the viral control is shown as not having undergone any deterioration this was

undiluted specimen however showed a slow decline in

These results indicate that a virus combined with minimal amounts of antibody does not undergo permanent alteration with respect to infectivity. The explanation for the gradual loss of acid reactivability under conditions of continuing virus antibody combination is presently not available. It can mean either that a nonacid reactivable complex had gradually developed or that acid dissociation still occurred, but that the infectious capacity of the virus had been irreversibly destroyed.

Fazekas *et al.*[12,13] have suggested that, in the course of washing monolayer cultures that had been inoculated with virus-serum mixtures, a certain amount



... after stand ng for varying periods of

of reactivation of neutralized virus will occur as a result of dilution dissociation. The net result of this effect is to indicate a higher survival than is in ... dilution, it was proposed, is the explana... fraction. An attempt was made to ...ting effect. A mixture of virus and ... was necessary prior to inocu... various numbers ... on the number of plaques developing except, perhaps, as a result of the first wash, and this effect is in the direction opposite to reactivation. In order to determine how much neutralized virus had adsorbed and how this quantity varied as a result of washing, the amount of neutralized virus was determined by acid reactivation

Here too it may be seen that no systemic change occurred as a result of washing

The above results are not consistent with the proposal that the adsorbed virus antibody complex is dissociable simply by dilution If this conclusion is accepted the question of the nature of the nonneutralizable fraction still remains unanswered Previously[4] it had been shown that the nonneutralizable fraction behaved unlike free virus in that the rate at which it penetrated into cells was slower and in that some of the virus could be neutralized by a serum containing antibody to the antiviral gamma globulin From these observations it may be inferred that the nonneutralizable virus had reacted with antibody but perhaps imperfectly so that adsorption and infection could still occur However to a varying degree infection could be aborted by the continuous presence of either antiviral antibody or antibody to the antiviral gamma globulin

TABLE 3

EFFECT OF WASHING ON THE NUMBER OF INFECTIVE AND ACID-REACTIVABLE VIRAL PARTICLES ADSORBED TO HELA MONOLAYERS AFTER INOCULATION WITH A VIRUS ANTISERUM MIXTURE

No of washes	(V AS)		(V)	
	Direct assay	Acid reactivable	Direct assay	Acid reactivable
0	90 3	2 170	—	—
5	49 3	800	70 7	4 7
10	52 3	1 200	—	—
1	36 3	570	102 0	—
3	25 0	370	109 0	1 7
5	33 3	800	104 0	—

Interaction Between Virus Antibody Complex and Host Cells

Thus far consideration has been given solely to the *in vitro* interaction between virus and antibody without consideration of the mechanism of neutralization In a previous report[4] it was shown that when monolayer cultures were inoculated with a virus-serum mixture the number of plaques that appeared could be influenced by the treatment of the cells prior to addition of the agar overlay If the cells were exposed to buffer of pH 4 8 as much as a fiftyfold increase in the number of plaques resulted compared with cultures treated with buffer of pH 7 Since these cultures had been washed prior to the treatment the conclusion to be drawn from this result is that virus-antibody complexes were adsorbed to the cells The effect of the acid buffer was to dissociate the antibody from the virus without simultaneously inducing the detachment of virus from the cell Studies on the quantitative aspects of this observation have suggested that one molecule of antibody will suffice to produce a complex capable of adsorbing to cells but unable to infect The inability to infect is however not absolute as indicated by the delay in the rate of appearance of plaques Cultures inoculated with virus serum mixtures show approximately a 14 per cent increase in the number of plaques on the fourth day

as compared with the third day, while cultures inoculated with free virus show about a 1 per cent increase This observation could mean that some of the adsorbed complexes either slowly penetrate as such or undergo reactivation prior to penetration

Summary

The interaction between virus and antibody leading to inactivation (that is neutralization) is a kinetic process under the control of nonvariable factors such as the size mass and number of reactive sites of the two reactants and variable factors such as temperature and concentration that affect either the frequency or the efficiency of the collisions between virus and antibody Under constant conditions and in the presence of excess antibody the number of collisions leading to inactivation will be constant per unit of time thereby resulting in first order kinetics of inactivation

Dilution of a virus antiserum mixture results in only a small degree (<1 per cent) of dissociation On the other hand if a virus antiserum mixture that has reacted at *p*H 7 is readjusted to *p*H 2 5, nearly quantitative dissociation occurs It is probably correct therefore to consider the virus antibody reaction as an equilibrium system that at *p*H 7 has such a high equilibrium constant that it is essentially irreversible at this *p*H

When virus that has combined with a small number of molecules of antibody is kept at 5° C under conditions that preclude further addition of antibody, it remains fully acid reactivable indicating that antibody induces no deterioration in the infective capacity of the virus Under conditions where the continuous addition of antibody occurs acid reactivability is gradually lost No explanation is available for this phenomenon

The inabil ty to neutralize the total population of a virus preparation has been shown to apply to several viruses including poliomyelitis WEE and NDV Although a precise explanation for this phenomenon is not yet available preliminary evidence indicates that the resistant virus has reacted

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ema n superficially adsorbed These observa ... sugg ... hy pothesis of the mechanism of neutralization A virus with 1 molecule of antibody attached to only 1 of the adsorption sites can either adsorb to a cell via a free site and produce an infection or adsorb to a cell via the antibody containing te without producing an infection in either case the adsorption is irreversible At the most elementary level the mechanism of neutralization is the inhibition of penetration by antibody either through simple steric h ndrance or through a more complex effect for example inhibition of viral enzyme activity[16]

Although the conclus ons of the present study are based upon an analysis of a single system several other investigations[8, 10, 16, 17] have resulted in obser

vations that are consistent with the present findings It is of course, inadvis able at present to offer the above hypothesis as the *modus operandi* of the neu tralization reaction for all viruses A system as complex as that composed of virus antibody host cell is capable of showing many variations in any one of its components It has been demonstrated, for example, that neutralizing an tibody obtained very early during immunization is qualitatively different from the very late antibody[19 20] It has been reported[1] that quite different re sults were obtained in neutralization studies of vesicular stomatitis virus de pending upon the host cells employed These results as well as those obtained in the earlier *in vivo* studies indicate that a critical examination of the neu tralization reaction in terms of the variability of each component of the system is now called for in view of the recent advances in the methodology of research in virology

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PROPERDIN AND THE INACTIVATION OF VIRUSES

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The discovery of properdin[1] has been considered by many to be the result of serendipity Instead, its isolation should be viewed as the result of observations by a prepared mind observations by one who had long been a student of complement and who understood well its properties, its functions, and its vagaries The reference is, of course, to the late Louis Pillemer The discovery and isolation of properdin were the direct result of experiments carried out by Pillemer and his colleagues in an attempt to purify C'3, the third component of complement [1]

These ingenious experiments led to the recognition that the adsorption of C'3 to zymosan at 37° C required an initial reaction between a distinct protein and zymosan before C'3 could react with the insoluble carbohydrate of yeast cell wall [2] The normal serum protein required for selective inactivation of C'3 was termed properdin Further experiments clearly demonstrated that properdin had a variety of biological activities, but that these could be expressed only in the presence of all components of hemolytic complement and the divalent cation, magnesium Properdin, the 4 components of complement, and magnesium were collectively termed the properdin system [?] Indeed, it was properly called a system because the absence of any single component ... other

responsible for any of the well recognized biological activities of fresh, normal serum This paper is concerned with a summary of results of experiments carried out to investigate the role of the properdin system in the inactivation of viruses Details of most of these studies have been published elsewhere [?]

Experimental

That fresh normal serum could inhibit the infectivity of certain viruses or potentiate the neutralization of others by specific antibodies has been known for a number of years [4-13] In 1949 it was demonstrated that normal fresh human, guinea pig, rabbit, and mouse sera contained a heat labile component that combined with and neutralized influenza A, influenza B, mumps, and Newcastle disease viruses [14] The reaction was temperature dependent and required divalent cations The data indicated that, whereas hemolytic complement was necessary for inhibition of these viruses, there was another heat-labile factor present in serum that combined with the virus and was essential for viral inactivation The evidence indicated that the heat-labile factor was protein [14] Other investigators have described similar findings [15-17] Descrip-

disease virus (NDV) and fresh human serum that contained no detectable heat stable neutralizing or hemagglutinin inhibiting substances were used The unheated sera employed reduced viral activity as measured by hemagglutination or infectivity, by 93 to 99 9 per cent Heated serum had no such effect

Neither serum from which properdin was adsorbed by zymosan (termed RP) nor properdin alone inhibited viral activity When purified properdin was added to an RP however the ability of the serum to inhibit viral hemagglutination or infectivity was restored All 4 components of complement as well as the divalent cation magnesium but not calcium were essential to inactivate NDV Only manganese could substitute for magnesium The concentration of manganese required for this function however was considerably in excess of that present in serum and therefore precludes the possibility that it might play a natural role in the activity of the properdin system These data implied that the properdin system was indeed responsible for inhibition of virus by fresh serum

The quantitative requirements for certain components of the properdin system as well as the milieu in which it functions were studied in an attempt to elucidate the mechanism of the viral inactivation described

Inactivation of NDV by the properdin system was shown to have marked temperature dependence There was no inhibition of the virus at 10° C or less, and between 10° and 37° C the quantity of virus inactivated was a straight line function related to temperature

Not only was magnesium required for inhibition but also the amount of virus inhibited was directly related to the concentration of magnesium present The ionic strength of the reaction mixture also markedly influenced the quantity of virus inactivated by the properdin system an ionic strength of 0 1 was required for maximum inhibition with ionic strengths of less than 0 1 or greater than approximately 0 15 viral inactivation was diminished Inhibition of NDV was also dependent upon the pH of the reaction mixture Maximum viral inhibition was obtained between pH 6 5 and 7 0, decreasing viral inactivation occurred on either the acid or alkaline side of this pH optimum

When measured by neutralization of infectivity or inhibition of hemagglutination the degree of viral inhibition was dependent upon the quantity of properdin added to a serum made deficient of this protein A smaller quantity could be detected by hemagglutination inhibition titrations (as little as 0 03 units of properdin) than could be measured by neutralization of viral infectivity

Although all components of complement as well as properdin were required for inactivation of the virus only properdin was reduced in concentration when a large quantity of virus was mixed with serum no reduction in any of the four components of hemolytic complement could be detected These findings correspond to those previously reported from investigation of the heat labile inhibitor of guinea pig serum in which the inhibitor was bound by influenza virus without a decrease in hemolytic complement titer [14] It might be proposed from these data that inactivation of NDV is accomplished by the combination of virus with properdin for inhibition of virus all components of complement are essential although they are not combined with the infectious agent in detectable quantities

To inactivate NDV by the properdin system, the divalent cation magnesium or manganese was required. Experiments indicated that removal of magnesium from the reaction mixture by the ion exchange resin amberlite IRC-50 in the sodium cycle resulted in dissociation and reactivation of 50 to 75 per cent of the inhibited virus, even when as much as 95 per cent of the total virus had been inactivated. This dissociation reaction was dependent on time and temperature. These data imply that magnesium is an important linkage in the properdin-virus combination, and that all of the virus inhibited is not irrevocably inactivated by the properdin system as measured by hemagglutination.

For a further understanding of the properdin system, the properties of properdin per se should be considered,[19] these are summarized in TABLE 1. Properdin comprises only a small fraction (less than 0.02 per cent), of the total serum proteins. In the earlier studies of the physical properties of properdin this protein was considered to be a beta 2 globulin. Following the suggestion of Deutsch and his co-workers,[19] electrophoretic characteristics indicate that a preferable terminology may be that of a gamma-1 globulin. By ultracentrifugation, purified properdin was found not to be homogeneous. However, properdin activity accompanied the particles having a sedimentation constant of 18 S. From these data the molecular weight was computed to be of the order of 1,000,000. The *isoelectric point of the protein was 5.6. Purified* [illegible]

[illegible]
molecular species.

TABLE 1

PROPERTIES OF PROPERDIN

[illegible]
[illegible]
[illegible]
Antigenic
Activity neutralized by antibody

There is substantial evidence available to indicate that the properdin system is capable of inactivation of several important viruses. TABLE 2 lists in the left column the agents that have been proved to be *inactivated by the properdin* system. Using children's sera that contained no specific antibodies for the viruses studied, it was possible to demonstrate that the properdin system neutralized influenza A and B viruses, as indicated by earlier studies of the heat labile serum inhibitor. Finkelstein and his co-workers have presented evidence to indicate that herpes simplex virus is inactivated by the properdin system.[20] The studies of Van Vunakis and her co-workers have clearly proved that the T2 bacteriophage can be inactivated by the properdin system,[21] and Wedgwood has demonstrated that T7 bacteriophage can be similarly inactivated

(unpublished data). Listed in the middle column of TABLE 2 are several viruses that are neutralized by heat labile factors in human or animal sera. The heat labile substance may act alone with some viruses or with others may act to potentiate the neutralization by antibody. In fact, neutralization of some strains of dengue virus by specific antibody requires the heat labile factor.[9] The neutralization of dengue virus suggests a situation similar to the neutralization of *Toxoplasma gondii* which requires specific antibody and the properdin system for inactivation of this infectious agent.[22, 23] The third column lists some important viruses known not to be inactivated by the properdin system either alone or with antibody. It would be intriguing to understand the basic differences between agents so sensitive to inactivation by the properdin system and those resistant to neutralization by these serum components. That the differences lie in the surface structure of the individual agents seems probable.

TABLE 2
INACTIVATION OF VIRUSES BY THE PROPERDIN SYSTEM

Viruses inactivated	Viruses probably inactivated	Viruses not inactivated
Newcastle disease	Mumps	Poliomyelitis
Influenza A	Vaccinia	Coxsackie
Influenza B	Variola	ECHO
Herpes simplex	Western equine	Adenoviruses
T2 bacteriophage	Rous sarcoma	
T7 bacteriophage	Dengue	

Conclusions

It is indeed an understatement to note that the properdin system is a complex one; an understanding of the mechanism of its action with respect to each of its component parts is a goal not yet approached. It is probable, however, that the factors that comprise this system, although dependent upon each other, act somewhat differently and independently. For example, the data described above imply that properdin combines with NDV, but that the essential complement components of the system are not reduced by the reaction that inhibits virus. It appears, however, that magnesium ions are an essential link for combination of properdin with virus. When magnesium is chelated, the complex is dissociated, at least partially, and virus is released.

Not only is it important to clarify the mechanisms by which the constituents of the properdin system exert their effect *in vitro*, but also to elucidate the pressing question of the biological role of properdin in the intact animal. There is a considerable body of evidence to suggest that the properdin system is implicated in a number of functions of natural resistance. To date, however, there is no unequivocal demonstration that increased susceptibility or resistance to a bacterial or viral infection is caused by alterations in properdin per se. Nevertheless, it must be pointed out again that the properdin system consists of six variables. To maintain one of these components as limiting without alteration of at least one of the others is a major hurdle. Thus, the complexity of the properdin system makes extremely difficult the rigid proof

that one component *properdin* plays a significant role in natural resistance. Indeed, evidence of the part that complement plays *in vivo* is still lacking. From a teleological viewpoint, however, it would seem unlikely that a complex system such as the properdin system that can lyse bacteria, kill protozoa, and inactivate viruses would exist in serum without some biological function.

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human serum from which properdin had been removed by adsorption with zymosan at 17° C (RP) In most studies human serum from which properdin and the third component of complement had been selectively removed by incubation with zymosan at 37° C (R3) was also added to increase the level of the second component of complement, which was usually the limiting factor in this human complement system These reagents were tested for properdin and complement activity before addition to the reaction mixtures and on several occasions reaction mixtures were tested after incubation to ascertain that neither complement nor properdin was significantly reduced during the one hour incubation at 37° C The LD_{50} virus dilutions were calculated by

TABLE 1
EFFECTS OF TREATMENT WITH X RAY CORTISONE ZYMOSAN AND PROPERDIN ON VIRUS INFECTIONS IN MICE*

Treatment	Expected *properdin* response	Log change in virus LD_{50}						
		Egypt intracerebrally			Mengo intracerebrally			Mengo intraperitoneally
None	No change	(1)	(2)	(3)	(1)	(2)	(3)	(4)
Properdin x2	rise	0 7	−0 4		−0 4	−0 3		
Properdin x4	rise							−0 1
X ray	fall		−0 4			0		
X ray and properdin x2	rise?		−0 7			0		
Cortisone	?	0 8			0 6			
Cortisone and properdin x2	rise	1 7			0 6			
Zymosan 10 mg /kg	rise			*0*			*0*	*0 1*
Zymosan 350 mg /kg	fall			0 2			0 2	0 1

* mice were

mice were

mice were

properdin

the method of Reed and Muench[16] Differences in LD_{50} titers in the several reaction mixtures were calculated from the simultaneous titer in the inactivated

17

of

titers Total complement titrations were performed using twofold serial dilutions of the test material with 1 cc of 1 per cent sensitized sheep red blood cells per tube and with visual estimation of the 50 per cent hemolysis end point

Treatment schedules for the *in vivo* experiments tabulated in TABLE 1 were X ray 150 r total body 6 days before virus properdin x2 schedule, 1000 U /kg intraperitoneally 4 hours before and 20 hours after virus x4 schedule 2000 U /kg 20 hours and 2 hours before and 20 and 44 hours after virus cortisone 125 mg kg intraperitoneally daily for 6 doses starting 48 hours

before virus, zymosan, 10 mg/kg or 350 mg/kg intraperitoneally (single dose) 20 hours before virus

The rationale of virus administration for the experimental treatment of advanced cancer of man and the resulting clinical, virological and serological reactions have been reported elsewhere [14, 19, 20] Viremia was determined by intracerebral inoculation of mice with heparinized blood taken daily for at least 14 days following virus inoculation

Results

In simultaneous studies the *in vitro* incubation of Mengo or Egypt 101 viruses in human serum with added complement or properdin or both caused no significant changes in virus titers from those found in reaction mixtures lacking both complement and properdin (TABLE 2) Likewise when the human serum

TABLE 2

LACK OF EFFECT OF PROPERDIN SYSTEM ON MENGO AND EGYPT 101 VIRUSES *IN VITRO* IN THE PRESENCE OR ABSENCE OF ANTIBODY

Reaction mixture			Logs of virus inactivated			
Reagents	Content of		Mengo virus I C		Egypt virus I C	
	Complement	Properdin	Pre serum	Anti serum	Pre-serum	Anti-serum
	u/ml	u/ml				
Serum* alone	0	0	†	2 7	†	[illegible]
Serum R1 R3	60	0	0 3	2 0	−0 1	[illegible]
Serum R1 R3 heated properdin	60	0	−0 1	2 0	0 3	[illegible]
Serum properdin	0	10	0 1	2 1	0 1	[illegible]
Serum R1 R3 properdin	60	10	0 5	2 2	−0 3	[illegible]
Serum R1 R3 properdin	60	100	0 1	2 0	0 4	[illegible]

* All sera inactivated at 56° C for 30 min

† Virus titers (intracerebrally LD₅₀ in mice) after incubation in * serum alone [illegible] 10 [illegible] West Nile 10 [illegible]

contained antibodies against these viruses complement and [illegible] no significant difference in the amount of virus neutralized [illegible] similar study using different antisera and complement plus [illegible] properdin for comparison with a complete properdin system [illegible] the same results

These studies seem to show conclusively that the properdin [illegible] virus inactivating effect on these two neurotropic viruses [illegible] to its virucidal effect on Newcastle disease virus and T2 [illegible] parallel studies with added antibody do not completely [illegible] that properdin may function as an accessory factor since [illegible] included in these experiments to determine whether [illegible] accessory factor would be active in this test system

The effects on virus infection of measures designed to [illegible] din levels of mice are presented in TABLE 3 Suscept[illegible] ured by determining the dilution of virus that killed 50 [illegible] various treatment groups as compared with untreated [illegible]

Zymosan administered at a dosage of 350 mg/kg or 10 mg/kg did not change the virus LD_{50}. The high dose of zymosan depressed properdin to subnormal levels for at least 24 hours, during which period the virus was administered, but was followed by a rebound *of properdin to above normal* (FIGURE 1). The low dose of zymosan caused a transient rise in serum properdin that did not persist beyond the period of virus inoculation.

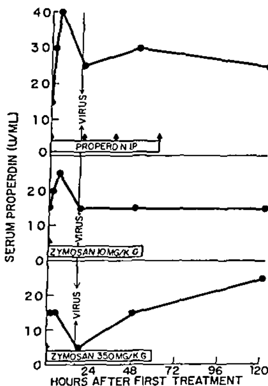


FIGURE 1. Effects of properdin or zymosan administration on serum properdin levels of mice. See text for details of dosage. All properdin titrations were done simultaneously with identical reagents. Each serum specimen was a pool from 3 mice.

The administration of human properdin to mice also failed to influence susceptibility to Mengo or Egypt 101 viruses. The x4 treatment schedule maintained serum properdin levels of the mice at twice normal levels throughout the course of the virus infection (FIGURE 1). Serial properdin levels were ... Treatment ...le change in ...go virus, and ...ise resistance ... time of virus ... reduction of serum properdin titer as compared with simultaneous untreated controls, and

the administration of properdin to such mice had restored properdin levels to normal, mice treated with cortisone plus properdin had a normal properdin level but the effect of cortisone treatment alone was not determined

Mean survival time of mice in all of these experiments was also unaffected by the several methods of treatment

Cancer patients undergoing infection by Egypt 101 virus as experimental chemotherapy for cancer untreatable by other methods showed no differences in time or duration of viremia related to their serum properdin levels This

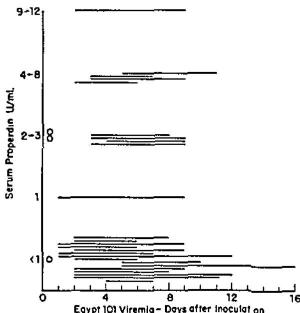


FIGURE 2 Initial serum properdin levels and pattern of viremia in 28 cancer patients treated with Egypt 101 virus Each line indicates days on which viremia was demonstrable in one patient Circles indicate no virus recovered

analysis included 28 consecutive patients whose inoculum was tissue-culture passaged virus (HEp 2 or HEp 3 cell lines) Properdin levels were determined on sera taken within one week prior to virus inoculation Data are presented graphically in FIGURE 2 Analysis of febrile response to virus infection likewise showed no discernible relationship to properdin levels (this virus caused no clinical reaction other than fever in this series of patients) Six patients treated with Mengo virus were studied in the same manner One patient had a properdin level of 8 U ml and had viremia on days 1 and 2 (that is in blood samples drawn 24 and 48 hours after virus inoculation) One had 1 U /ml and viremia on day 1 only 4 had no detectable properdin (>1 U /ml) and of these 2 had viremia on day 1 only one on days 1 to 3 and the other on days 1 to 4 These data concerning Mengo virus infections are not sufficient to per-

m t any conclus ons but they do not suggest any relationship between pro perdin levels and susceptibil ty to Mengo virus

Summary

The properdin system did not inactivate West Nile (Egypt 101) or Mengo viruses on incubation *in vitro* nor did it enhance the virus-neutralizing effect of specific antisera. Treatment designed to lower or to raise the serum proper din levels in mice had no discernible effect on susceptibility to infection by these viruses. In man variations of serum properdin levels did not appear to influence the course of these same virus infections as judged by duration of viremia or by clinical course

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STUDIES OF THE NONSPECIFIC INACTIVATION OF NEWCASTLE DISEASE VIRUS BY MAMMALIAN SERA*

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The inactivation of Newcastle disease virus (NDV) by fresh serum has been demonstrated to involve the components of the properdin system, namely, properdin, complement (C′), and magnesium.[1,2] Interest in the reaction be [illegible]

[illegible]

bears upon the question of the possible "specificity" of properdin or the potential multiplicity of properdins.[3,4] There is also interest in the kinetics of the inhibitor virus system as an example of biological inactivation and a comparison of it with classic specific antibody.

The purpose of this presentation is to review our experience with certain biological aspects of the NDV inhibitor system. In the latter portion of the paper we present preliminary data concerning the development of a plaque assay technique that permits experimental conditions analogous to those recently described for the properdin phage system.[6,7]

Viral Inactivating Substance (VIS) versus NDV Measured in the Chick Embryo

Initial studies concerning the inactivation of NDV by fresh mammalian serum were made in the chick embryo.[8] The system may be described briefly as follows: Undiluted fresh serum was mixed in equal volume with undiluted [illegible]

in parallel. The VIS(NDV) titer was defined as the amount of virus inactivated, expressed as the log LD_{50}. This was determined by the difference in titer between the heat inactivated control and the unheated serum following the 1 hour incubation period. Using this system, the VIS titers were found to be species-characteristic. Human sera titered 4.0 to 4.5, rabbit sera 2.5 to 3.0, and chicken sera 0.0 to 0.5. The effect of the time and temperature of incubation upon VIS activity is demonstrated in FIGURE 1. Aliquots of fresh whole human serum mixed with 8.0 log LD_{50} of virus were maintained at 3 temperatures: 0°, 22° and 35° C. Samples of the reacting mixtures were removed at indicated times, immediately placed at 0° C and titered for residual infectious virus. At 0° C the inactivation was negligible even after 120 min. At 22° C 2.4 log LD_{50} were inactivated at 60 min. and 3.5 log LD_{50} at 120

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min At this temperature the reaction curve may not have been complete at 120 min At 35° C the reaction proceeded rapidly, especially during the first 5 min It was essentially complete at 60 min, when 4 5 log LD_{50} were inactivated One hour and 35° C were selected as standard reaction conditions.

The effect of the initial virus input upon the observed VIS titer was studied (FIGURE 2) Inputs varying from 5 to 8 log LD_{50} were mixed with fresh whole

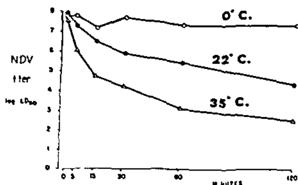


FIGURE 1 The effect of time and temperature on VIS(NDV) titer Ten volumes of fresh whole human serum were incubated with 1 volume of NDV allantoic stock virus at the indicated temperatures (average of 2 experiments)

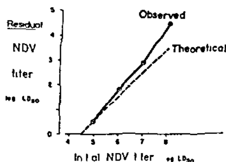


FIGURE 2 The effect of virus input on VIS(NDV) titer One volume of fresh whole serum was incubated with an equal volume of virus (average of 5 experiments)

serum In general the amount of virus inactivated was proportional to the input, although at the highest concentration which was undiluted allantoic fluid there was a decrease in viral inactivation The theoretical residual NDV, were it to follow the percentage law without deviation would fall as indicated on the dashed line The reduced VIS titer found with the use of undiluted allantoic virus was not altered when the virus was partially purified by washing or red blood cell adsorption and elution

It has been observed that, following a 2 hour incubation in the presence of fresh serum, a residual population of NDV remains viable This residue is a constant percentage of the initial population over a partial range This

"steady state" may have a number of explanations, these have been discussed by others in connection with antibody neutralization systems [9,10] Certain factors have been explored in the VIS-NDV system In order to learn whether the steady state is due to depletion of the VIS of fresh serum, the following experiment was performed (FIGURE 3) Undiluted human serum (10 volumes) was incubated with 8 2 log LD_{50} of NDV (1 volume) at 35° C for 1 hour An aliquot was sampled for residual virus At this time an amount of NDV equal to the initial input was added to the original mixture and the total was incubated for another hour and again assayed A third cycle of incubation following re-addition of NDV was then performed It may be seen that the addition of a large quantity of NDV to the reacting mixture on 2 successive occasions resulted each time in the reduction of virus titer for 8 2 to 3 1 or 3 2 log LD_{50} Thus, despite the fact that the reacting mixture at equilibrium contained approximately 1000 infectious units, the serum present is capable

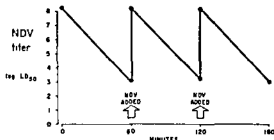


FIGURE 3 The effect of adding NDV to VIS-virus mixture at equilibrium Ten volumes of fresh whole serum were incubated with 1 volume of NDV ($10^{8.2}$ LD_{50}) At 60 and 120 minutes 1 additional volume of NDV containing $10^{8.2}$ LD_{50} of virus was added to the mixture (average of 3 experiments)

of inactivating 100,000,000 infectious units of freshly added virus Thus, under the conditions of this experiment, VIS was in excess in the original reaction mixture Addition of fresh serum to the mixture at equilibrium in an amount equal to the original serum volume did not decrease significantly the residual live virus Egg passage of virus that remained infectious after treatment with VIS at each passage level did not yield an NDV line with increased resistance to VIS

When the experiment described in FIGURE 3 was repeated, using a lower ratio of serum to virus, the results obtained were quantitatively different (FIGURE 4) One volume of fresh undiluted human serum was incubated with

essentially no loss of activity due to serum incubation alone The apparent depletion of VIS may be due to specific action of NDV upon the VIS system

NDV acted similarly to whole allantoic virus Normal allantoic fluid had little or no inhibitory effect Whether this VIS inactivation is due to the virus infectious unit itself or to cellular products could not be distinguished in these experiments

Inhibition of VIS Effect

VIS may be inhibited by a variety of procedures, these are summarized in TABLE 1 Assuming the VIS system to be a complex one, the procedures may nullify the activity of VIS in diverse manners, and these have been discussed *in a prior publication*[8] *Any of these treatments can inhibit completely the*

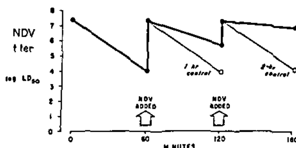


FIGURE 4 The effect of adding NDV to VIS virus mixture at equilibrium Equal volumes of serum and virus were used Other conditions as in FIGURE 3 (average of 2 experiments)

TABLE 1
INHIBITORS OF HUMAN VIS(NDV)

Heat	56° C for 30 min
Streptokinase	1000 units/ml
Trypsin	100 mg %
EDTA	0 2 M
Bacteria *E coli* 026	10^9
Tissue	whole suspension

VIS(NDV) activity of fresh whole human serum Special comment is indicated regarding tissue components When a serum virus mixture was inoculated into the chick embryo prior to completion of the reaction, the reaction was stopped Similarly treatment of the embryo with VIS prior to virus inoculation resulted in no antiviral activity These observations suggested the possibility of VIS inhibitors in chick embryo tissues (TABLE 2) It was found that the chick embryo and chorioallantoic membrane reduced the VIS titer, while allantoic fluid, yolk, and albumin did not Furthermore, certain mammalian tissues were potent inhibitors of VIS The tissue inhibitor was stable at 56° C for 30 min, and partial activity remained at 100° C for 30 min The tissue inhibitor was associated with the larger sedimentable cell fractions, nuclei, mitochondria, and microsomes The identity of the tissue component is unknown, but a comparison may be drawn with the polysaccharide inhibitors of properdin described by Pillemer *et al*[11,12]

Attempts to Reactivate Virus in VIS Virus Mixtures

It would be important to learn whether the NDV VIS combination could dissociate under suitable circumstances yielding infectious virus One can take advantage of the fact that the VIS system may be inactivated by gentle means means that do not destroy viral infectivity In a series of experiments trypsin streptokinase guinea pig kidney and ethylenediamine tetra acetate (EDTA) were used to treat a virus VIS mixture in an attempt to recover infectious virus In no case was there significant evidence of release of virus Dissociation was also attempted using simple dilution It has been shown that a log log plot of the slope relating virus inactivation to VIS dilution is steep that is approximately 3 5[13] Since the slope exceeds 1 0 it would be expected if spontaneous dissociation occurred that virus could be recovered from a noninfectious mixture upon dilution Such release did not take place The use of high ionic strength pH 7 4 buffer by Pillemer[14] for elution of proper

TABLE 2
TISSUE INHIBITORS OF VIS(NDV)

	Active	Inactive
Chicken	Whole embryo Chorioallantoic membrane Spleen Brain Kidney	Allantoic fluid Albumin Yolk
Mammalian	Kidney Lung Spleen Brain Liver Muscle	Red blood cells

din from its substrate zymosan suggested its use for viral reactivation No virus release was observed when the NDV VIS mixture was diluted in such a buffer when compared to appropriate controls Also the release of virus did not occur when large amounts of heat inactivated NDV were added to a noninfectious mixture Thus many of the methods of reactivation technique used in the study of the dissociability of the virus antibody complex failed to indicate recovery of free virus Further work should be done along these lines before we accept the concept that VIS irreversibly binds or destroys viral infectivity Wedgwood *et al* have described the reactivation of NDV hemagglutinin under special conditions[2]

In addition to inactivation of infectivity other properties of NDV have been inactivated by VIS The ability of NDV to function as an interfering agent was studied in the following manner An interfering system was set up between fully infectious virus of 2 strains of NDV in the chick embryo taking advantage of the lack of virulence of the Blacksburg strain for this host It was found that undiluted (10^8 LD_{50}) Blacksburg strain inoculated into the allantoic sac would routinely prevent death due to strain B (10 to 32 LD_{50})

inoculated 4 hours later The experiments were terminated on day 5 since deaths due to Blacksburg virus occurred after this time Treatment with fresh serum of the Blacksburg virus nullified its ability to protect against the lethal B infection. Heat inactivated serum *did not destroy the interfering property* of the Blacksburg strain.

Similarly the effect of VIS upon the neurotoxic effect of NDV in the mouse was studied Strain Cg was found to be capable of causing a neurological illness and death within 2 to 4 days following intracerebral inoculation of high titers of virus. The virus did not multiply, and the effect was not transmissible Fresh serum incubated with the virus eliminated the neurotoxicity Quantitative titrations revealed that the degree of inactivation of virus infectivity was proportional to the reduction of neurotoxic effect. VIS failed to influence the neurotoxic effect when administered in the contralateral hemisphere simultaneously or prior to introduction of virus or when inoculated into the peritoneal cavity *in vitro* incubation of VIS with virus was required

In summary 4 different properties of NDV are known to be inactivated by VIS. Infectivity *interference, and neurotoxicity have been discussed* In addition inactivation of the hemagglutinin has been shown [2] At present it may be assumed only on quantitative grounds that the same mechanism simultaneously destroys all of these viral modalities

VI_{50}(NDV) Assayed by Plaque Inhibition

The shortcomings of the use of the chick embryo and the LD_{50} in studying VIS are apparent in the lack of accurate quantitation and the inability to manipulate experimental conditions We have recently initiated experiments using the inhibition of plaques on the chick fibroblast monolayer as an assay system *The VI_{50}(NDV) unit is defined as the serum concentration that* will inhibit 50 per cent of the input plaque forming units (pfu) under standard conditions Titers are expressed as VI_{50} units/ml of undiluted serum The relatively heat stable Kansas strain was used to minimize spontaneous degrada

a buffer for serum and virus dilutions was required to fulfill the following requirements in addition to an appropriate ionic strength and pH (1) adequate protection against spontaneous virus degradation during 37° C incubation (2) avoidance of any serum serum component or tissue extract that would enhance or inhibit VIS activity (3) high buffering capacity, and (4) adequate concentration of magnesium *A Veronal buffered saline containing magnesium* ions (5×10^{-4} M)[15] and with the addition of 0.5 per cent lactalbumin hydrolyzate was found to be satisfactory *

$$\log x = \log K + 1/n \log (y/1 - y)$$

* Since this paper was prepared for [illegible] been observed that lactalbumin hydrolyzate is capable of inh[illegible] VIS act[illegible] *of 1 per cent crystalline bovine* [illegible]bumin is now used in its [illegible]

in which x is the serum concentration and y is the fraction of virus inactivated A plot of log x versus log $(y/1 - y)$ yields a straight line in the partial range with a slope $1/n$ and intercept log A The VI_{50} value is obtained at the intersect for 50 per cent viral inactivation $(y/1 - y = 1)$ In the example given, the VI_{50}/ml of unheated human serum was 100 with a slope of 0 39 The

TABLE 3
VI_{50} (NDV) IN HUMAN SERUM VON KROGH CALCULATIONS

	Unheated					Virus control	Heated		
Final serum dilution	60	80	100	120	140		4	10	20
Serum conc per ml (x)	0 017	0 012	0 010	0 008	0 007		0 250	0 100	0 050
Average pfu	32	49	75	87	104	142	53	77	114
Fraction inactivation (y)	0 77	0 66	0 47	0 39	0 27		0 63	0 46	0 20
$y/(1 - y)$	3 35	1 94	0 89	0 64	0 37		1 70	0 85	0 25
Slope ($1/n$)	0 39						0 90		
VI_{50}/ml	100						7		

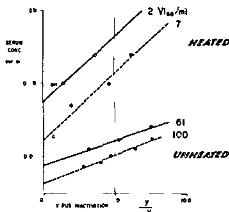


FIGURE 5 A von Krogh plot of 2 human sera unheated and heated to 56° C for 30 min The slope values ($1/n$) for unheated sera are 0.39 (serum C) and 0 35 (serum D H) for heated sera 0 90 (serum C) and 0 92 (serum D H)

same serum heated at 56° C for 30 min had a VI_{50} ml of 7 units and a slope of 0 90 The von Krogh plots of this serum along with a second example, are shown in FIGURE 5 The greater slope values and lower titers of the heated sera are demonstrated

Two or more tests on aliquots of sera stored at −70° C were performed over a period of several weeks The reproducibility obtained is indicated in TABLE 4 The titers of unheated sera A B, and C were approximately 100 units, and of D approximately 50 units The slopes varied from 0 37 to 0 51

The heated sera had titers varying from 1 unit to 44 units, and the slope values varied from 0.7 to 1.4. TABLE 5 presents similar figures for 3 infants, 3 to 9 months of age. The general similarity of the values for infants to those of adults is of interest. Further work is indicated to establish whether VIS titers fall following birth, as is true of passively acquired transplacental antibodies. The low unit values of the heated sera should be noted. Adult values

TABLE 4
REPLICATION OF VI_{60} AND SLOPE (1/N) OF HUMAN SERA

Human sera	Unheated		Heated	
	VI_{60}/ml	$1/n$	VI_{60}/ml	$1/n$
A	100	0.42	44	0.80
	104	0.37		
B	87	0.49	33	1.3
	120	0.52		
C	80	0.37	8	0.69
	96	0.42		
	100	0.39	5	1.4
			7	0.96
	110	0.51	5	0.76
D	48	0.37	<1	
	52	0.46	4	0.70
	53	0.41	2	

TABLE 5
VI_{60} (NDV) AND 1/N IN UNHEATED AND HEATED INFANT AND ADULT HUMAN SERA

Human sera	Unheated		Heated	
	VI_{60}/ml	$1/n$	VI_{60}/ml	$1/n$
Infants	61	0.35	2	0.92
	80	0.54	4	1.0
	89	0.26	2	0.76
Adults FG	143	0.27	54	0.65
RB	310	0.63	112	0.98
Pool	153	0.31	32	0.85

presented in the lower portion of the table illustrate individuals with higher unheated VI_{60} values. Serum RB,* with a titer in unheated serum of 310 units, *also demonstrated* a very high heat stable value, namely, 112 units. This high heat-stable VI_{60} may be reflected in the high $1/n$ value (0.63) of the unheated serum. The last line presents values for a serum pool prepared from 24 adult donors. The slope values for human sera may be compared to those obtained for the inactivation of T2 phage by Barlow *et al.*,[7] who obtained slopes of 0.37 ± 0.1, using a system similar to that described here. The mean

* Initials of donor

of 10 human sera in the present study was 0.41. TABLE 6 gives the values obtained with animal sera. The mean VI_{50}/ml value for 7 individual rabbit sera was 32; for 5 guinea pig sera, 67. Heated VI_{60} of rabbit sera were high in relation to the unheated titers. The slope value of the heated sera was higher than the unheated, generally approaching 1.0. Most chicken sera studied had high heated VI_{50} values. Two chickens with little or no heat stable activity had unheated VI_{50} values which were quite low: 11 and 16.

Let us consider this system as a possible bio-assay for properdin as defined by the zymosan system. Apparently, there are several limitations.

The presence of heat stable viral inactivating substance in almost all individual human and animal sera tested was an unexpected finding, although

TABLE 6

VI_{50} (NDV) AND 1/n IN UNHEATED AND HEATED ANIMAL SERA

Animal sera	Unheated		Heated	
	VI_{50}/ml	1/n	VI_{50}/mL	1/n
	17	0.51	4	0.50
	19	0.56	5	0.94
	28	0.43	10	0.81
Rabbit	30	0.54		
	32	0.67	26	1.2
	43	0.66	21	1.3
	54	0.41	19	1.2
mean	32	0.54	14	0.99
	42	0.54	5	0.68
	59	0.45	21	0.60
Guinea pig	71	0.36		
	74	0.46		
	85	0.38		
mean	67	0.44		

in many individuals the quantity was of a low order. The stable substance differed from the unheated serum in that the slope values in general were closer to 1.0. An attempt was made to influence the level of heat stable material by immunizing rabbits with related myxoviruses, influenza and mumps. Such

viruses that have been shown recently to be ubiquitous in man and animals.[17,18] Following immunization with NDV the labile and stable titers appeared to augment each other. This is unlike the situation with bacteria where there is an inhibition of the bactericidal activity of the properdin system by specific bacterial antibodies.[11]

The unheated VI_{50}/ml titers obtained in human serum by the NDV assay are higher than the unit values obtained by either the zymosan or phage assay methods. The unheated VI_{50} end points with the Newcastle assay frequently

exceed the whole C′ titers in human sera, although not the titers of individual C′ components When the VI₅₀ values obtained for human rabbit and guinea pig sera were compared to the normal values of whole C′ for these species ... Proper l n assays employ ng an RP reagent

were made available to us by J L Barlow, H Van Vunakis,* and L Levine † This RP preparation had a hemolytic titer of 70 per cent of the original value of the untreated serum pool (Barlow, personal communication) Incubation of the RP with zymosan resulted in less than a 20 per cent reduction of the hemolytic titer, indicating little residual properdin The RP had less than 10 per cent of the untreated serum T2 phage neutralizing capacity When tested in the NDV plaque inhibition system, this RP preparation showed only a modest reduction in VI_{50} titer between the untreated serum pool and the RP prepared from it However, there was an increase in the slope value of the RP as

TABLE 7
INHIBITION OF VIS BY ANTIHUMAN PROPERDIN ANTISERUM

		Average pfu	Fraction inactivation
Human serum 1/60 (2 VI_{50} units)	+ Diluent	13	0 87
	+ Antihuman properdin (A) antiserum	106	0
	+ Normal rabbit serum	14	0 86
Human serum 1/60 heated	+ Diluent	110	0
Virus control		100	

compared to the original pool value, the significance of this change is not clear It appears logical to explore the possibility that removal of properdin from serum by zymosan may differentially affect the ability of the serum to inac ... phage and NDV The resistance of anti NDV activity to zymosan

the possibility that phage inactivation is associated wi ... properdin [5]

The relationship between t ... strated in another manner with RP (anti HP[A]) [‡] prep ... activity of human serum (TA ... dilution 1/50) were incubated ... 1/40) at 4° C for 18 hours The virus fraction inactivated fell from 0 86 in

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‡ Reagents kindly furnished by Barlow Van Vunakis and Levine

the untreated serum to 0 in the antibody treated serum The antibody titer, expressed as the number of VI_{50}(NDV) units inactivated by 1 ml of antiserum was greater than 80 This is in agreement with measurement of activity of

fact that whole serum may include factors other than the properdin system that may play a role in the inactivation of virus The demonstrated antiviral activity of such serum may not be a direct function of the properdin level as assayed by the zymosan technique The term inactivation is preferred to neutralization because the latter has been associated with the reaction of

heat labile activity in serum in terms of the substrate, for example NDV T2 phage T7 phage bacteria, or zymosan

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INTERFERENCE OF NEWCASTLE DISEASE VIRUS WITH NEUROPATHOGENICITY OF ONCOLYTIC VIRUSES IN MICE*

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Introduction

Among the criteria by which we judge the activity of a given virus is its ability to cause a characteristic disease. One method of suppressing this type of biological activity is to expose the normally susceptible cells, tissue, or organism to another virus, often dissimilar in biological nature. Although such interference between viruses has been known for many years,[1,2] it remains poorly understood, and the term as we use it may well embrace several different mechanisms.[3,4] This interference is an interesting and, in some cases, a remarkably effective method of biologically suppressing the pathogenicity of viruses.[5]

Many oncolytic viruses are also neurotropic.[7,8] This dual tropism, while interesting in itself, presents a problem in utilizing these viruses in the laboratory and is a theoretical hazard of real concern that severely limits the clinical use of such viruses as a form of experimental therapy.[10]

We present here an attempt to use Newcastle disease virus (NDV) to interfere with the neuropathogenicity of three oncolytic viruses, Mengo, Bunyamwera, and the Egypt 101 isolate of West Nile virus in normal and in tumor-bearing mice, with the objective of producing a systemic oncolytic infection and then using viral interference to prevent the usual ensuing viral encephalitis.

Materials and Methods

The NDV used was the Massachusetts strain in egg passages 137 and 139. It was stored as frozen aliquots of infected allantoic fluid in a dry ice cabinet. Mice were injected intracerebrally (I.C.) using 0.03 ml of the undiluted infected allantoic fluid. This dosage caused no mortality. Normal allantoic fluid (NAF) was harvested from eggs of the same shipment used to grow NDV stocks.

The Egypt 101 isolate of West Nile virus was in tenth passage and Bunyamwera virus was in seventh passage, both in mouse brain. Mengo virus was also in mouse brain, but of unknown passage history. These 3 viruses were stored in small aliquots in a dry ice cabinet as 20 per cent mouse brain suspensions in 5 per cent rabbit serum in 0.15 M NaCl containing 100 U penicillin and 0.5 mg streptomycin per milliliter. Further dilutions were made in 5 per cent horse serum in 0.15 M NaCl with penicillin and streptomycin as above. For titrations, 5 consecutive tenfold dilutions were injected into groups of five 18- to 20-gm Swiss Webster mice, using 0.03 ml I.C. or 0.05 ml intraperitoneally (I.P.), using those dilutions expected to include the LD_{50}.

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† Postdoctoral Fellow of the Public Health Service.

as judged from previous tests Mice dying within 24 hours of injection were discarded Others were inspected every day for paralysis and death in those experiments in which dates of death are considered, and every weekday in other experiments Virus LD_{50} dilutions were calculated from deaths through the fourteenth day

The Ehrlich carcinoma, ascitic form (EC
K Sugiura and routinely maintained by
intervals of 7 to 10 days [11] For experim

TABLE 1

INTERFERENCE BY NDV WITH BUNYAMWERA, EGYPT 101, AND MENGO VIRUSES IN NONTUMOR BEARING MICE

Neurotropic virus and route	Interval between treatment and neurotropic virus	Log of reciprocal of LD_{50}		
		Treatment after I C. NDV	Controls	
			After I C. NAF	No prior treatment
Egypt 101 I C	2 hrs	5 4	7 2	6 8
	1 day	4 7	>7 5	>7 5
	2 days	4 8	7 4	7 0
	3 days	5 4	6 9	6 9
	4 days	4 7	7 0	6 8
Bunyamwera I C	2 hrs	6 4	7 6	7 3
	1 day	5 8	7 1	7 2
	2 days	6 0	7 2	7 4
	3 days	6 2	7 6	7 5
	4 days	5 2	7 0	7 0
Mengo I C	1 day	7 0	7 8	7 1
	2 days	7 4	7 6	7 4
	3 days	6 7	7 4	6 8
Mengo I P	2 hrs	6 5	7 4	7 1
	1 day	5 5	6 8	6 6
	2 days	6 7	6 5	7 2
	3 days	7 1	7 0	7 4
	4 days	7 1	7 4	6 8

RESULTS

Newcastle Disease Virus versus Intracerebral Egypt 101 Virus

When Egypt 101 virus was titrated I C in mice from 2 hours to 4 days after I C inoculation of NDV, the LD_{50} of Egypt 101 was consistently decreased by 1 4 to 2 8 logs as compared to its LD_{50} in NAF-inoculated controls or untreated controls (TABLE 1) This represents absolute protection of NDV-treated mice against a mean of somewhat over 100 LD_{50} of Egypt 101 There

was no consistent difference in the LD_{50} obtained in the two types of control groups FIGURE 1 represents combined daily cumulative mortality data from Egypt 101 titrations done 2 hours and 2, 3 and 4 days following NDV (I C) and their controls These data were pooled because titrations performed within each of the 3 major treatment groups consistently showed the same pattern of mortality The difference in end point between NDV treated mice and the 2 types of controls reflects the level of absolute protection afforded by NDV, but mice treated with NDV which were not completely protected died at the same rate as controls This is shown more clearly (FIGURE 2) when only mice dying of infection during the titrations period are considered and their

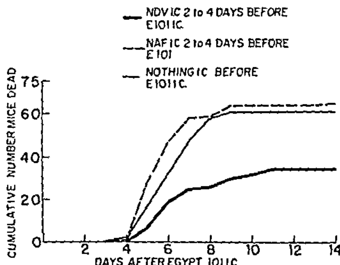


FIGURE 1 Cumulative mortality of mice used for I C titration of Egypt 101 virus 2 hours to 4 days after I C NDV

rate of death calculated as percentage of mortality against time Mice from the 1-day titrations were not included because of the lack of a suitable end point in the control groups however their pattern of cumulative mortality did not differ from those of the groups presented

Similarly there was no absolute protection and little difference in cumulative mortality in another experiment in which a very large inoculum (20 000 LD_{50}) of Egypt 101 virus was injected I C 24 hours after I C NDV (FIGURE 3)

Newcastle Disease Virus versus Intracerebral Bunyamwera Virus

When Bunyamwera virus was titrated intracerebrally 2 hours to 4 days after I C NDV the LD_{50} of Bunyamwera was decreased 0 9 to 1 8 logs as compared to its LD_{50} in untreated or NAF injected controls (TABLE 1) This shows less absolute protection than was obtained against Egypt 101 In addition to the absolute protection there was a delay of deaths in the NDV treated mice

(FIGURE 4) This result is in contrast with those obtained with the Egypt 101 virus This prolongation of survival is seen more clearly when only mice that died of infection during the observation period are considered and their daily cumulative mortality calculated (FIGURE 5)

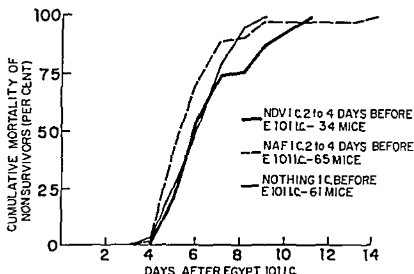


FIGURE 2 Absence of delay in mortality of mice not surviving I C titration of Egypt 101 virus

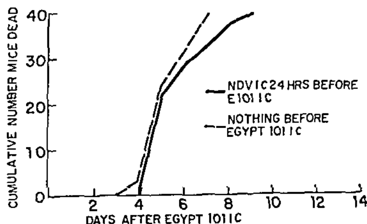


FIGURE 3 Absence of effect of prior I C NDV on mice receiving 20 000 LD_{50} Egypt 101 virus I C

This pattern of delay in death was also seen in another experiment in which 40 mice were injected I C with a large dose (30 000 LD_{50}) of Bunyamwera virus 24 hours after receiving I C NDV The rate of mortality differed markedly from that of controls that did not receive NDV (FIGURE 6) and a few

of the NDV treated mice survived Mice in a group set up in parallel with this experiment were sacrificed on several successive days, and their blood and brains were tested for virus There was less recoverable Bunyamwera virus in both blood and brains of mice protected by NDV (TABLE 2)

In another experiment in which a very small dose of Bunyamwera (30 LD_{50}) was injected I C in groups of 40 mice, the mice that received I C NDV 24

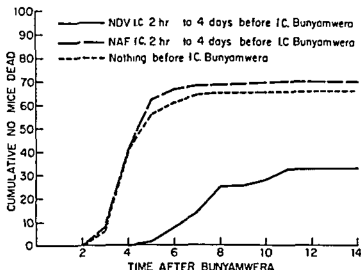


FIGURE 4 Cumulative mortality of mice used for I C titration of Bunyamwera virus 2 hours to 4 days following I C NDV

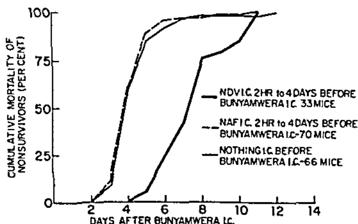


FIGURE 5 Delayed mortality of NDV treated mice not surviving I C titration of Bunyamwera virus

hours before the Bunyamwera virus had only 10 per cent mortality by day 14 in contrast to 98 per cent mortality by day 5 in the non NDV treated controls (FIGURE 7) However, mortality continued to increase slightly even during the third week Virus isolation studies on groups of mice set up in parallel

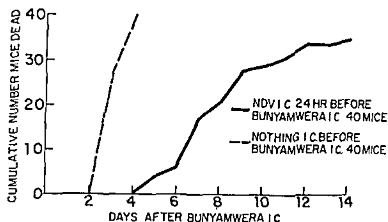


FIGURE 6 Delayed mortality of NDV treated mice receiving 30 000 LD_{50} Bunyamwera virus

TABLE 2
EFFECT OF NDV ON RECOVERY OF BUNYAMWERA VIRUS FROM BLOOD AND BRAIN*

Bunyamwera inoculum (LD_{50})	Tissue	Treatment	Virus recovery days after inoculation									
			1		2		3		4		5	
30 000	Brain	NDV	—		1 5	3 3	1 5	3 5	0 7	3 5	4 5	>5 5
30 000	Brain	Control	—		>5 5	>5 5	5 2	>5 5	—	—	—	—
30 000	Blood	NDV	—		0/3	1/3	0/5	0/3	0/5	3/5	0/5	
30 000	Blood	Control	—		5/5	5/5	5/5	3/3	—	—	—	—
30	Brain	NDV	<0 5	<0 5	<0 5	0 7	<0 5	0 7	0 7	0 7	—	
30	Brain	Control	3 5	4 5	5 3	>5 5	>5 5	>5 5	>5 5	>5 5	—	
30	Blood	NDV	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	—	
30	Blood	Control	1/5	0/5	5/5	3/4	5/5	3/3	4/4	5/5	—	

* Figures for brain indicate the virus titer (log ID_{50}) figures for blood indicate the number of mice dead over the total number injected with undiluted blood

with this experiment also showed less virus in blood and brain of the NDV treated mice (TABLE 2)

Newcastle Disease Virus versus Intracerebral and Intraperitoneal Mengo Virus

When Mengo virus was administered I C or I P within 24 hours after I C NDV there was delay in paralysis and death (FIGURES 8 to 11), but LD_{50} titers differed by less than one log which indicates no absolute protection (TABLE 1) When Mengo was inoculated (I C or I P) 48 or more hours after NDV, there was no significant delay in deaths

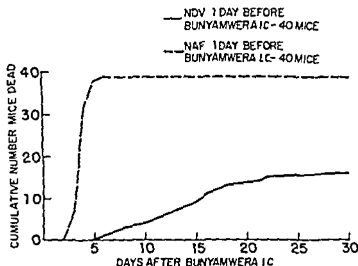


FIGURE 7 Absolute protection and delayed mortality in mice receiving 30 LD_{50} Bunyamwera virus following NDV

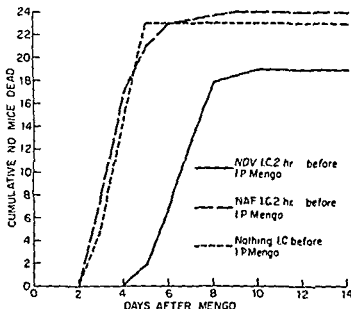


FIGURE 8 Delayed mortality in mice used for I.C. titration of Mengo virus within 24 hours after I.C. NDV *See* FIGURES 10, 11 and 12

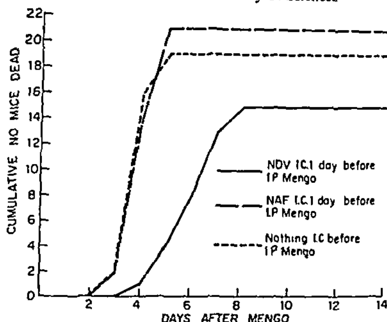


FIGURE 9 Delayed mortality in mice used for I C titration of Mengo virus within 24 hours after I C NDV *See* FIGURES 9, 11, and 12

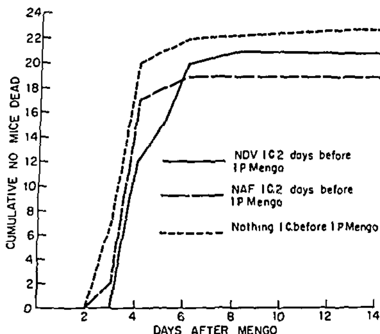


FIGURE 10 Delayed mortality in mice used for I C titration of Mengo virus within 24 hours after I C NDV *See* FIGURES 9, 10, and 12

Several schedules of multiple I C injections of NDV were investigated, but none was more effective in delaying death than a single injection of NDV

Newcastle Disease Virus versus Egypt 101 Virus in Mice Bearing Ehrlich Ascites Tumors

I P Egypt 101 virus does not consistently cause paralysis and death in adult mice even when 0 5 ml of a 20 per cent infected mouse brain suspension is injected Mice implanted I P with 1 to 2 million Ehrlich ascites cells become uniformly susceptible to paralysis and death following I P Egypt 101 virus

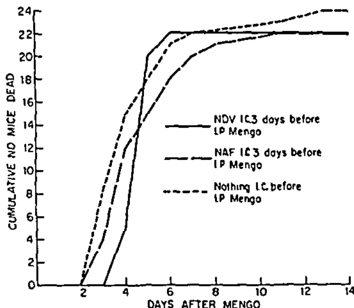


FIGURE 11 Delayed mortality in mice used for I C titration of Mengo virus within 24 hours after I C NDV See FIGURES 9 10 and 11

When Egypt 101 was given I P 1 day after implantation of Ehrlich ascites cells it was possible to differentiate deaths due to Egypt 101 virus from deaths due to the Ehrlich ascites alone (FIGURE 12) Deaths due to I P Egypt 101 in ECI implanted mice occurred within 10 days were often preceded by hind quarter paralysis and occurred in mice that had not developed overt ascites NDV I C did not influence such virus deaths Deaths due to the Ehrlich ascites generally occurred after the tenth day were not preceded by hind quarter paralysis and were preceded by marked ascites As judged by these criteria the pattern of mortality of mice receiving different concentrations of Egypt 101 virus I P was not influenced by treating mice with I C NDV (TABLE 3) NDV I C without subsequent I P Egypt 101 did not alter the pattern of mortality following implantation of Ehrlich ascites ECI cells incubated at 56° C for 30 min did not render mice susceptible to similar concentrations

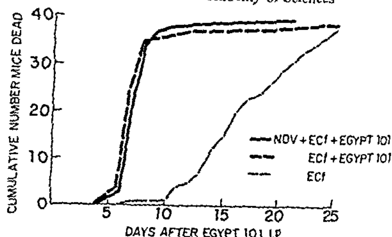


FIGURE 12 Cumulative mortality of mice receiving I P Egypt 101 virus 1 day after implantation of 1 6 × 10^6 Ehrlich cells, indicating absence of effect of NDV on such mortality and separation of virus mortality from ECf mortality

TABLE 3
PATTERN OF MORTALITY OF NORMAL AND TUMOR BEARING MICE RECEIVING NDV I C AND EGYPT 101 VIRUS I P

Treatment	Dilution of virus	Dead		Surviving Day 21
		Day 1 to 10	Day 11 to 21	
NDV (I C) 24 hours before, and ECf 2 hours before E101 virus I P	10^{-3}	4	1	0
	10^{-4}	2	3	0
	10^{-5}	0	5	0
	10^{-6}	0	5	0
	10^{-7}	0	5	0
ECf 2 hours before E101 virus I P	10^{-3}	5	0	0
	10^{-4}	2	3	0
	10^{-5}	1	4	0
	10^{-6}	0	5	0
	10^{-7}	1	4	0
Heated ECf 2 hours before E101 virus I P	10^{-2}	0	0	5
	10^{-3}	0	0	5
	10^{-4}	0	0	5
	10^{-5}	0	0	5
	10^{-6}	0	0	5
E101 virus I P only, no ECf	10^{-2}	0	0	5
	10^{-3}	0	0	5
	10^{-4}	1	1	3
	10^{-5}	0	0	5
	10^{-6}	0	0	5
NDV 22 hours before ECf	—	0	5	0
ECf only	—	0	5	0
Heated ECf only	—	0	0	5

of Egypt 101 virus I P No significant group of mice survived both Egypt 101 virus and the Ehrlich carcinoma

Newcastle Disease Virus versus Bunyamwera Virus in Mice Bearing Ehrlich Ascites Tumors

Bunyamwera virus is not consistently lethal for mice when administered I P The implantation of 1 to 2 million ECf cells rendered mice susceptible to death from the Bunyamwera virus However, a variable percentage of such mice given I P Bunyamwera virus survived their virus infection and failed to

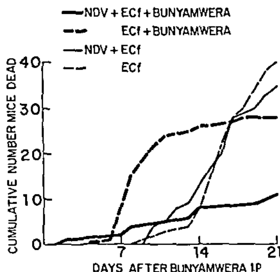


FIGURE 13 Cumulative mortality of mice receiving I P Bunyamwera virus 1 day after implantation 1 8 × 10[6] Ehrlich cells indicating the increased survival of mice receiving both I C NDV and I P Bunyamwera

develop ascites tumors They ... pelvic tumors Although man by many weeks, almost all of gradually expanding solid abdominal tumors

When NDV was administered I C to such tumor bearing mice prior to a large I P inoculum of Bunyamwera virus, there was a marked reduction in deaths due to virus and ascites rarely occurred (FIGURE 13) The surviving mice, however, did develop solid tumors that eventually caused death Thus it was possible, by suitable manipulation of the experimental situation, to palliate the course of disease caused by the Ehrlich carcinoma in the mouse by means of an oncolytic virus and to increase significantly the frequency of palliation by interfering with the neuropathogenicity of the oncolytic virus

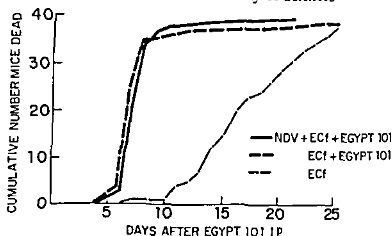


FIGURE 12 Cumulative mortality of mice receiving I P Egypt 101 virus 1 day after implantation of 1 6 × 10⁶ Ehrlich cells indicating absence of effect of NDV on such mortality and separation of virus mortality from ECf mortality

TABLE 3
PATTERN OF MORTALITY OF NORMAL AND TUMOR BEARING MICE RECEIVING NDV I C AND EGYPT 101 VIRUS I P

Treatment	Dilution of virus	Dead		Surviving Day 21
		Day 1 to 10	Day 11 to 21	
NDV (I C) 24 hours before and ECf 2 hours before E101 virus I P	10^{-3}	4	1	0
	10^{-4}	2	3	0
	10^{-5}	0	5	0
	10^{-6}	0	5	0
	10^{-7}	0	5	0
ECf 2 hours before E101 virus I P	10^{-3}	5	0	0
	10^{-4}	2	3	0
	10^{-5}	1	4	0
	10^{-6}	0	5	0
	10^{-7}	1	4	0
Heated ECf 2 hours before E101 virus I P	10^{-2}	0	0	5
	10^{-3}	0	0	5
	10^{-4}	0	0	5
	10^{-5}	0	0	5
	10^{-6}	0	0	5
E101 virus I P only no ECf	10^{-2}	0	0	5
	10^{-3}	0	0	5
	10^{-4}	1	1	3
	10^{-5}	0	0	5
	10^{-6}	0	0	5
NDV 22 hours before ECf	—	0	5	0
ECf only	—	0	5	0
Heated ECf only	—	0	0	5

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Discussion

These studies demonstrate interference between virus combinations not previously investigated, but do not further elucidate the mechanisms of viral interference. It may be pertinent to note that the degree of protection offered by NDV to Egypt 101, Bunyamwera, and Mengo viruses is inversely related to the rapidity of their lethal action in mice. This is consistent with a previous

NDV treated mice is consistent with previous observations in animal systems.[6,12]

The varying pattern of absolute protection and delay of death produced by NDV against these neuropathogenic viruses is as yet not explained.

The increased susceptibility of tumor bearing animals to oncolytic viruses has been noted before in this and other systems.[9,13] Whether this results from suppression of defense mechanisms in tumor bearing animals or from increased virus propagation in the neoplastic cells has not been established. However, the facts that there is a concentration of virus (presumably due to propagation) in the neoplastic tissues and that in the present studies the effect was produced by a relatively small number of tumor cells implanted only a few hours before virus inoculation favor the latter hypothesis, although it is consistent with both.

Although previous studies have demonstrated both the oncolytic capacity of Bunyamwera virus and the ability of NDV to interfere with certain neurotropic viruses, the present study is unique in its demonstration that the interference phenomenon can be used to suppress an undesirable consequence of a viral infection (neuropathogenicity) without affecting another and desirable effect (oncolysis) of the same infection.

Summary

(1) Prior I.C. injection of NDV protected mice against approximately 100 LD_{50} of Egypt 101 virus inoculated I.C., but did not affect the course of disease in those that succumbed. NDV protected against small multiples of the LD_{50} of Bunyamwera virus I.C. and also increased survival time in the mice that died. NDV increased survival time in mice inoculated with Mengo virus I.C. or I.P. if given within 24 hours, but gave no absolute protection.

(2) Mice with Ehrlich ascites usually succumbed to infection by Egypt 101 and Bunyamwera viruses inoculated I.P., whereas normal mice are highly resistant to these viruses by the I.P. route.

(3) In tumor bearing mice the efficacy of NDV interference against Egypt 101 virus was not demonstrable.

(4) Bunyamwera virus palliated the course of Ehrlich carcinoma, and such palliation was significantly increased by the use of NDV to interfere with the neuropathogenic effect of the Bunyamwera virus infection.

Acknowledgments

We gratefully acknowledge the technical assistance of Carol Jacobs, Terry Krulwich, and Virginia Babcock in these studies.

2 5 ml volumes, including the virus at titers of 10^9 to 10^{10} pfu/ml, representing physical particle concentrations at about 10^{11} to 10^{12}/ml, and formaldehyde at a concentration of 3 mM (1 4000 formalin) or as designated were used The appropriate formaldehyde concentrations were prepared by dilution from stock solutions whose concentrations were determined by the colorimetric procedure of West and Sen (1956), which in turn was standardized by a gravimetric procedure (Yoe and Reid, 1941) In those experiments employing the tracer, $HC^{14}HO$ was included at a level of 2 µc/ml or as designated Unless otherwise indicated, the only other constituents of the reaction mixtures were water and sodium phosphate or pyrophosphate buffer at 0 01 M and pH 7 0 the pH having been previously adjusted with HCl To minimize any mixing effects, the reaction mixtures were prepared in an ice bath, mixed, zero time samples removed, and then quickly warmed to incubation temperature Small rubber stoppered test tubes were employed and incubation was performed in a water bath without stirring at 37° C or at the designated temperature

Samples for bioassay were removed from the reaction mixture at zero time and at various intervals, the sample usually being 10 λ (0 010 ml) immediately diluted into 0 99 ml of medium 199 The diluted samples were stored on ice until further diluted and assayed the storage period generally being not greater than 2 days Assay was by a modification (Fogh and Lund 1955) of the plaque technique using monkey kidney monolayer cultures in Petri dishes (Dulbecco and Vogt 1954) or prescription bottles (Hsiung and Melnick, 1955) Preliminary experiments showed that 1 4000 formalin in the inoculum, a concentration tenfold or greater than that in most assays, did not interfere with plaque formation or counts

Radioactivity of solid samples in metal dishes was measured in a gas flow counter Total $HC^{14}HO$ was measured by precipitation along with carrier formaldehyde in the counting dishes by the addition of an excess of dimedone in a pH 4 5 acetic acid ammonium acetate buffer followed by drying in a desiccator Samples usually 0 2 to 0 3 ml for assay of combined $HC^{14}HO$ were removed from the reaction mixture at appropriate times and dialyzed at 4° C against 3 changes of 0 02 M pyrophosphate buffer at pH 9 to remove free and reversibly bound formaldehyde The dialyzed samples were transferred to microcuvettes and weighed to determine the volume The ultraviolet absorption (Schwerdt and Schaffer 1956) was measured and the samples were then transferred quantitatively to counting dishes To avoid polymerization and retention of any residual free formaldehyde, about one third volume of methanol was added to each sample prior to drying in a desiccator in the presence of methanol vapor The mass of material present in the dried samples of either the total or bound $HC^{14}HO$ was insufficient to necessitate self absorption corrections

Inactivation

If inactivation of a virus by formaldehyde is due to a chemical reaction between formaldehyde and some site essential for infectivity the laws of bimolecular reactions should be followed Since formaldehyde is ordinarily employed in great excess, first order kinetics yielding a straight line on a semilogarithmic

INTERACTION OF HIGHLY PURIFIED POLIOVIRUS WITH FORMALDEHYDE*

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Information concerning the interaction of formaldehyde with a virus is not only important for the practical aspect of vaccine preparation, but can be useful in the fundamental elucidation of viral structure and mode of action The inactivation of poliovirus by formaldehyde as well as by other agents has been reviewed elsewhere (McLean and Taylor, 1958, Schaffer and Schwerdt, 1959) Previous reports of the use of purified or partially purified poliovirus in formaldehyde inactivation studies have included those of Charney and his co-workers (1957) and of Lycke (1958*a*) The nature of the chemical reactions involved in the interaction of poliovirus and formaldehyde has not as yet been reported The present studies have shed light on both facets of the problem, namely, biological inactivation and chemical reaction, the latter accomplished by the use of C^{14} labeled formaldehyde The use of highly purified virus for inactivation studies was desirable to avoid possible complications imposed by proteins and other substances of host tissue or culture medium origin For obvious reasons, direct chemical studies necessitate purified starting material or purification of the virus formaldehyde reaction product

Materials and Methods

[illegible]

Calif For most of the experiments [illegible] technique previously described (Schwerdt and Schaffer, 1956), with substitution of glycerol for sucrose in density gradient sedimentation Glycerol was removed from the highly purified product, fraction D, by dialysis against 0 01 M phosphate or pyrophosphate buffer at *p*H 7 In a few experiments virus purified by an alternate procedure (C F T Mattern and F L Schaffer, unpublished data) involving ammonium sulfate precipitation, 2 cycles of high and low-speed ultracentrifugation, and ultracentrifugation in a CsCl gradient (Meselson *et al*, 1957) of mean density 1 34 was employed with no significant differences in results

With the aid of microchemical techniques a small volume of concentrated [illegible]

* The work reported in this paper was supported in part by a grant from the National Foundation New York N Y

obtained w th crude tissue culture flu d (Lycke 1958b) An example of an experiment on inactivat on at various pHs is presented in TABLE 2

The effect of various substances upon inactivation of poliovirus was studied The rate of inactivation for crude unfiltered tissue culture fluid and for partially

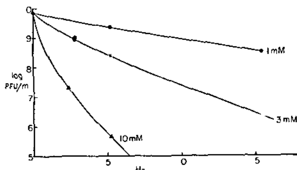


FIGURE 4 Effect of formaldehyde concentration on inactivat on of purified MEF 1 pol o virus at 37° C and pH 7

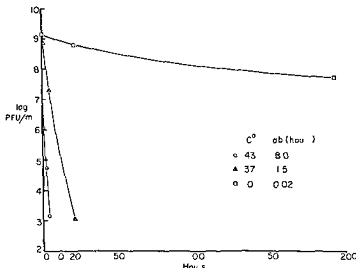


FIGURE 5 The effect of temperature on inactivation of purified Saukett poliov rus w th 3 mM HCHO at pH 7

pur fied virus (the product $SP_N^{2\nu e}$ after 2 cycles of ultracentrifugation—*see* Schwerdt and Schaffer 1956) was not outside the range of *ab* values obtained with highly purified virus These tests were made under the comparable con d t ons of small volume and no st rring The addition of salt (1 2 M NaCl) tissue culture medium 199 or a low concentrat on of protein (0 01 per cent

tained An example of such an experiment is presented in FIGURE 4 In this experiment the values for the product *ab* divided by the millimolar formaldehyde concentration were 0 20 0 22 and 0 32 at 1, 3 and 10 mM, respectively Although not studied in detail variation of virus concentration over a one-hundredfold or one thousandfold range appeared to have little or no effect upon the inactivation rate

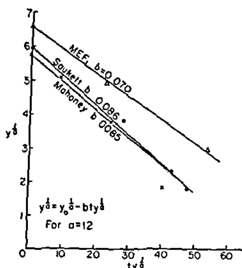


FIGURE 3 The same data as in FIGURE 2 plotted as $y^{\frac{1}{a}}$ versus $ty^{\frac{1}{a}}$ for the value $a = 12$

TABLE 1
A SUMMARY OF INACTIVATION DATA FOR 3 STRAINS OF HIGHLY PURIFIED POLIOVIRUS WITH 3 mM HCHO pH 7 AND 37° C*

Virus	No	ab (hr)	
		Mean	Range
Mahoney	9	0 93	0 7 1 3
MEF 1	4	1 11	0 7 2 0
Saukett	6	1 18	0 9-1 4
All 3 strains	19	1 04	0 7 2 0

* Inactivation rates are expressed as the product of parameters *a* and *b*

As expected a marked effect of temperature upon formaldehyde inactivation of purified virus was observed The results of such an experiment are presented in FIGURE 5 It was not possible to obtain an Arrhenius plot from this or other experiments carried out at various temperatures but it would appear that the energy of activation would be of the order of one half or less that of the 20 to 28 kcal /mole reported for crude material by Lycke (1958b)

The effect of *p*H upon inactivation of purified virus was not marked in the region of neutrality the results being essentially in agreement with the results

obtained with crude tissue culture fluid (Lycke, 1958b) An example of an experiment on inactivation at various *p*Hs is presented in TABLE 2

The effect of various substances upon inactivation of poliovirus was studied The rate of inactivation for crude unfiltered tissue culture fluid and for partially

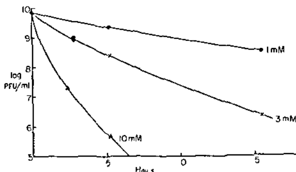


FIGURE 4 Effect of formaldehyde concentration on inactivation of purified MEF-1 polio virus at 37° C and *p*H 7

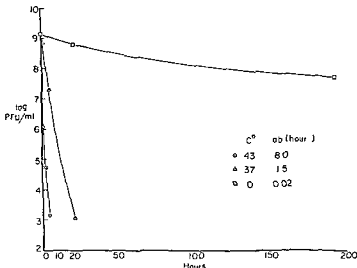


FIGURE 5 The effect of temperature on inactivation of purified Saukett poliovirus with 3 mM HCHO at *p*H 7

purified virus (the product, SP_N^{2uc}, after 2 cycles of ultracentrifugation—*see* Schwerdt and Schaffer, 1956) was not outside the range of *ab* values obtained with highly purified virus These tests were made under the comparable conditions of small volume and no stirring The addition of salt (1 2 M NaCl), tissue culture medium 199, or a low concentration of protein (0 01 per cent

bovine serum albumin) had little if any effect upon inactivation of highly purified virus Addition of an equimolar concentration of $NaHSO_3$ completely blocked inactivation by formaldehyde The effect of relatively high concentrations of other substances that combine with formaldehyde is discussed below

The phenomenon of delayed appearance of plaques or cytopathic effects with poliovirus partially inactivated with formaldehyde has been observed by various investigators (Schultz *et al*, 1957, Böttiger *et al*, 1958) In the present investigation plaque counts at 4 days were appreciably higher than at 3 days with partially inactivated preparations whereas only small increases in counts were experienced with untreated virus Plaques continued to appear until the plates or bottles were discarded due to degeneration or confluence of plaques but the increase over the count at 4 days was ordinarily insufficient to alter significantly the results over the range of inactivation studied

Binding of Radioactive Formaldehyde

The problem of chemical reactions and their relation to inactivation of purified tobacco mosaic virus (TMV) with formaldehyde was approached many

TABLE 2
THE EFFECT OF *p*H UPON INACTIVATION OF PURIFIED MEF 1 POLIOVIRUS WITH 2 4 mM HCHO AT 37° C

	Log pfu/ml
Initial titer	9 8
Titer at 19 hrs	
*p*H 6	5 1
*p*H 7	6 3
*p*H 8	5 7

years ago by Ross and Stanley, (1938) using chemical techniques More recently, radioactive formaldehyde has been used in studies of inactivation and chemical binding with purified TMV and its isolated ribonucleic acid (Staehelin 1958) More extensive studies of the uptake of $HC^{14}HO$ by TMV have been made by Meriwether and Rosenblum (1957, 1959) The latter authors observed a rapid initial uptake of formaldehyde followed by a prolonged linear increase in the amount of formaldehyde associated with the particles

Irreversible chemical reactions should be of greater interest than reversible reactions in relation to inactivation since inactivation of poliovirus is presumably not readily reversible (*see below*) Preliminary experiments revealed that extensive dialysis of the virus $HC^{14}HO$ mixture against a slightly alkaline buffer yielded minimal C^{14} counts with minimal losses or effects upon the infectivity and physical integrity of the particles For that reason dialysis against pyrophosphate buffer at *p*H 9 was adopted as the standard procedure and for the purposes of these experiments the formaldehyde remaining after such treatment may be defined as irreversibly bound For the calculation of the number of molecules of formaldehyde bound per average physical particle it was assumed that there was no change in the extinction coefficient at 260 mμ As indicated below this assumption is probably not valid with extensive treatment

of the virus with formaldehyde It serves over the range of most of the studies and will be used for the lack of a better practical quantitative measure of particle concentration

The results for the binding of formaldehyde by purified poliovirus were qualitatively quite similar to the results of Meriwether and Rosenblum with TMV in that a slow continual increase was observed following a rapid initial uptake The results of 4 separate experiments with the 3 virus types are presented in FIGURE 6 From these and other similar experiments it was obvious that the

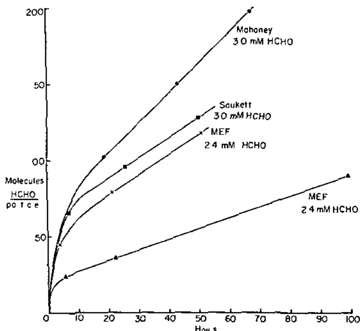


FIGURE 6 Binding of C^{14}-labeled formaldehyde by poliovirus plotting the calculated number of HCHO molecules bound per physical particle against time

uptake was not highly reproducible from experiment to experiment but that in any single experiment the same form of curve was obtained With reaction periods of 6 to 10 days several hundred molecules were combined per particle and apparently the uptake was continuing The maximum calculated uptake was 1300 molecules per particle for a sample in 8 mM HCHO for 118 days but as previously pointed out the assumption of particle count based upon optical density was not valid in such a case

In an experiment to determine the form of the early portion of the formaldehyde uptake curve the usual conditions were employed except that the C^{14} level was increased to 6 μc/ml and $NaHSO_3$ was added in equimolar amounts prior to dialysis of the samples The rapid uptake portion of the curve ap

peared to be linear up to at least 7 hours The binding at 1 hour, corresponding to inactivation of about one half the virus, was approximately 10 to 12 formaldehyde molecules per particle

The effect of formaldehyde concentration upon binding by the particles was that of a direct relationship TABLE 3 shows the effect of concentration upon the binding of formaldehyde at a specified time and also indicates that at that time only one molecule was bound per 5700 formaldehyde molecules present in the mixture

An example of the effect of *p*H upon the uptake of formaldehyde is shown in TABLE 4 The binding was considerably greater at *p*H 8 than at *p*H 7, while the calculated binding at *p*H 6 was only slightly higher than the uptake at *p*H

TABLE 3

THE EFFECT OF FORMALDEHYDE CONCENTRATION UPON BINDING OF FORMALDEHYDE BY PURIFIED MEF 1 POLIOVIRUS AT *p*H 7 AFTER 17 HOURS INCUBATION AT 37° C

HCHO conc mM	HCHO combined/particle	HCHO total/combined
1	13	5.5×10^3
3	37	5.8×10^3
10	126	5.7×10^3

TABLE 4

THE EFFECT OF *p*H ON FORMALDEHYDE UPTAKE AND ULTRAVIOLET ABSORPTION ON PURIFIED MEF 1 POLIOVIRUS AFTER 5 DAYS INCUBATION IN 2.4 mM HCHO AT *p*H 7 AND 37° C

	HCHO uptake *molecules/particle*	Ultraviolet absorption OD_{260}/OD_{280}
*p*H 6	129	1.54
*p*H 7	95	1.69
*p*H 8	240	1.72
Orig virus	—	1.72

7 It is to be noted that a change occurred in the ultraviolet absorption spectrum in the *p*H 6 sample leaving the calculation open to question and posing a problem as to what is happening to the gross structure and chemical nature of the particles during treatment with formaldehyde This latter will be discussed below The marked increase in formaldehyde uptake with increase in *p*H may be taken as an indication that a predominant reaction is dependent upon charge of a dissociable group

Reduction of the temperature of the reaction mixture has revealed a decreased uptake of $HC^{14}HO$ by the particles Because of the complex kinetics and lack of precise data at various temperatures the effect cannot as yet be quantitated

To obtain information regarding the physical nature of virus particles after reaction with formaldehyde a sample of purified Saukett virus was incubated 6 days at 37° C with 13 mM HCHO including C^{14}, at a level of 15 μc/ml A 0.25 ml aliquot of the mixture was layered over a glycerol density gradient

(including pH 8 pyrophosphate buffer) in a 2 ml tube and centrifuged under conditions comparable to those previously used in purification of the virus. A light scattering band was observed at or slightly above the region in which a virus band is usually found. A considerable amount of light scattering material was also observed toward the top of the tube, suggesting breakdown products of smaller size. The fractions were dialyzed against pyrophosphate buffer in the usual manner, and the ultraviolet absorption and $HC^{14}HO$ uptake were determined. The results are shown in FIGURE 7. In spite of the appearance which suggested an unequal distribution of material, ultraviolet absorption indicated appreciable material in each fraction. Radioactivity, however, was not associated equally with all fractions. Since the bottom portion of the tube contained no more radioactivity or ultraviolet absorbing material than expected due to drainage, there was no indication of appreciable aggregation of particles. The optical density ratios of the various fractions suggest that the top portion was richest in nucleic acid. From the position and ultraviolet spec

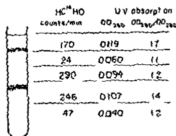


FIGURE 7. Schematic representation of appearance and results after sedimentation of purified Saukett virus after 6 days of incubation at 37° C with 13 mM HCHO labeled with C^{14} at a level of 15 μc /ml

trum of the major band it is tempting to associate it with the noninfectious particles of fraction C separated during purification. The results of this single experiment suggest that treatment with a relatively high concentration of formaldehyde causes physical disruption of the particles.

Effects of Substances Combining with Formaldehyde, and Reactivation Attempts

Early reports by Ross and Stanley indicated partial reactivation of formaldehyde treated TMV by subsequent treatment at pH 3. Almost complete reactivation of formaldehyde inactivated bacteriophage by the use of certain amino acids and other substances reacting irreversibly with formaldehyde was reported by Heicken and Spicher (1956). It was of interest to test such procedures with purified poliovirus partially inactivated with labeled formaldehyde. TABLE 5 shows the results of an experiment in which MEF 1 virus was inactivated for 18 hours under the usual conditions of concentration, pH, and temperature. Samples were then dialyzed in the cold against buffers at various pH values, after which infectivity titers and C^{14} counts were obtained. There was no evidence for reactivation at any pH tested, but it is seen that the acidic

conditions favored the retention of formaldehyde that could be dialyzed away under neutral or slightly alkaline conditions

In another experiment the effects of tryptophan one of the amino acids binding formaldehyde irreversibly, and glycine, an amino acid that combines with the reagent only reversibly, were tested during inactivation and subsequent dialysis of Mahoney strain virus Glycine has commonly been employed in inactivation of poliovirus by Swedish workers (Wesslen *et al*, 1957, Lycke

TABLE 5

THE EFFECT OF DIALYSIS AT VARIOUS *p*H VALUES UPON INFECTIVITY AND HC HO BINDING OF PURIFIED MI F 1 POLIOVIRUS AFTER 18 HOURS INCUBATION WITH 3 mM HCHO AT *p*H 7 AND 37° C

	Log pfu/ml	HCHO molecules pa t e
0 Hour	9 7	—
18 Hours	5 0	—
18 Hours dial at *p*H 3	4 3	295
18 Hours dial at *p*H 6	4 8	215
18 Hours dial at *p*H 7	5 0	85
18 Hours dial at *p*H 9	5 1	71

TABLE 6

THE EFFECTS OF GLYCINE AND TRYPTOPHAN ON INACTIVATION AND ON INFECTIVITY AFTER SUBSEQUENT DIALYSIS OF PURIFIED MAHONEY POLIOVIRUS

	0 01 M PrO₇	0 01 M P O₇ + 0 02 M Glyc ne	0 01M PrO + 0 005 M T yptophan
0 Hour	9 5	—	—
2 Hours	9 0	9 1	9 1
5 Hours	8 5	8 5	8 8
21 Hours	6 2	6 5	7 8
Dialyzed 4° C	—	6 7†	7 8‡
Room temp	6 5*	6 2†	7 9‡
Room temp	6 6‡		

° C D alysis was at approximately

1958*a*) The infectivity data shown in TABLE 6 reveal a protective effect somewhat delayed in its onset, of tryptophan and no appreciable effect of glycine in confirmation of the bacteriophage studies (Heicken and Spicher 1956) In contrast to marked reactivation of bacteriophage dialysis of the partially inactivated poliovirus against tryptophan had no appreciable effect The $HC^{14}HO$ remaining after further dialysis was approximately the same for all the samples

In preliminary experiments it was found that Tris (Tris hydroxymethyl aminomethane) buffer particularly in its uncharged alkaline form, formed a non dialyzable sedimentable polymer with formaldehyde This of course made the buffer unsuitable for the tracer studies When Tris was present in appre

ciable concentration, for example 10 mM the effect during inactivation was similar to that observed with tryptophan

Discussion

The results of these studies are in general agreement with those of the Swedish investigators and appear to be compatible with their interpretations involving modification of the virus particles as reactions with nonessential sites proceed Since these are discussed more extensively elsewhere in this monograph by Gard only some of the points more pertinent to the present study will be mentioned here

In contrast to departure from first order kinetics of inactivation with purified material in the present work and that of Lycke (1958*a*), first-order kinetics were observed by Charney *et al* (1957) The reason for the discrepancy is not apparent but it may lie in differences of techniques employed in inactivation or assay

It may well be possible that biological inactivation of a virus can be effected either by truly *inactivating its genetic material the nucleic acid or by blocking its entry into or release of its nucleic acid within the host cell* In the latter case, the particle could still be potentially infectious and infection could result if the proper conditions were provided Reactivation such as that of bacteriophage (Heicken and Spicher 1956) could be simply the removal of a reversibly bound agent from the site of a highly specific attachment mechanism Such effects would appear to be minimal if at all existent, with formaldehyde and poliovirus as indicated by the present studies and by the common use of bisulfite to stop the reaction On the other hand the delayed appearance of infection in tissue cultures with partially inactivated preparations might be due to some such mechanism Although adsorption and penetration of the virus probably proceed normally release of the nucleic acid to initiate infection might be partially blocked by an altered protein coat

As shown by the tracer studies binding of formaldehyde by poliovirus particles appears to involve two or more mechanisms or binding sites Diffusion was proposed as the controlling mechanism for similar results with TMV (Meriwether and Rosenblum, 1957, 1959) If diffusion were not the limiting mechanism rapid saturation of certain readily available sites followed by a slower but continuing reaction with other sites might account for the observed uptake curves The slowly reacting sites could presumably be made available by the breaking of hydrogen bonds or alteration of protein or nucleic acid configuration

A large number of sites on each particle do react with formaldehyde but the relationship between reacting sites not essential for infectivity and those causing true inactivation is not readily apparent from current data Certain inferences may be drawn however Ribonucleic acid is known to react with formaldehyde and if infectious to be inactivated by this reagent (Fraenkel Conrat 1954 Staehelin 1958) The amino groups of the bases appear to be the reaction sites Thus the presumption that inactivation of poliovirus is due to reaction with the genetically important ribonucleic acid portion of the particle has some basis The observation that inactivation is relatively independent of pH in the region of neutrality as compared with the marked effect upon

$HC^{14}HO$ uptake would suggest that the essential sites do not dissociate in this region. The amino groups of the nucleic acid bases are essentially uncharged at neutrality.

Of various possible reactions of proteins with formaldehyde, binding to amino groups followed by cross linking (Olcott and Fraenkel-Conrat, 1947) would appear to be the only important reaction occurring under the mild conditions employed in inactivation. It would seem that, if cross linking of protein groups were involved in the reaction, the particles should be more stable, but the results of the density gradient experiment would indicate the contrary. As a possible explanation, cross linking within protein subunits might weaken those bonds responsible for the macromolecular organization of protein subunits and nucleic acid of the whole particle. The reactions of the protein of the particle have implication in regard to the antigenic nature of the inactivated material, but, since studies of their antigenic nature were not included in the present investigation, they will not be discussed here.

The techniques employed in this work are not comparable to those used for vaccine production, and thus one should not attempt to carry over the results *in toto* to such systems. The information gained in the study of highly purified virus interactions with formaldehyde should prove useful, however, in the design of experiments and interpretation of results in other fundamental studies as well as in the practical aspects of the preparation of vaccines. Many of these results must be considered preliminary in nature because of limited data available in some cases and because of the lack of quantitative reproducibility from experiment to experiment. Nevertheless, they do point out various fundamental aspects of the problem and should aid in the further elucidation of those aspects.

Summary

Highly purified poliovirus has been employed in studies of inactivation by formaldehyde and chemical binding of formaldehyde. First order kinetics were not observed for inactivation, but the inactivation rate decreased with time. Chemical binding studies also revealed complex kinetics, with a rapid initial uptake followed by a prolonged continuing increase. The influence of various conditions upon inactivation and formaldehyde binding is presented, and possible interrelations between chemical binding and inactivation are discussed.

Acknowledgments

My colleagues and associates at the University of California have been most helpful in these investigations. I especially wish to thank W. M. Stanley for his continued interest and encouragement and C. E. Schwerdt, with whose collaboration these studies were begun. I also express my appreciation to R. Haschemeyer and H. Fraenkel-Conrat for their helpful discussions, and to Joan Blake, Helen Fisher, Ruth Sills, and Mary Parker for their excellent technical assistance.

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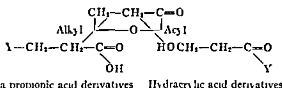
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INVESTIGATIONS OF THE USE OF BETA-PROPIOLACTONE IN VIRUS INACTIVATION*

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Beta propiolactone (BPL) has been found to inactivate effectively all of the viruses against which it has been tested These include Eastern and Western equine encephalitis (EEE and WEE), the MM strain of encephalomyocarditis (MM virus), Theiler (TO) encephalomyelitis, rabies (CVS), poliomyelitis (I, II, and III), ECHO (4 and 9), Coxsackie (B-3 and B 5), herpes simplex New Jersey vesicular stomatitis, adenovirus-3, measles, mumps, hog cholera, influenza (A and B), hepatitis, and *Escherichia coli* T 3 phage In addition, BPL has been found to be bactericidal and fungicidal The efficacy of beta propiolactone† as a "cidal" agent lies in the stability of the lactone form of the drug in its purified and concentrated state, together with its instability in a water solution The lactone ring opens at both the alkyl and acyl bonds, making it



Beta propionic acid derivatives   Hydracrylic acid derivatives

highly reactive to a wide variety of substances What has been most interesting to us has been its ability to react with all of the radicals associated with proteins[1 6] The biological products sterilized with beta-propiolactone have been proved to be free of drug toxicity and allergic reactions

Most of our studies have been, and continue to be, directed toward the sterili

What was of equal importance was to find an agent that would sterilize blood components and still maintain them safe to administer intravenously Of 623 chemical agents screened, 23 likely virucides were studied Of these, beta propiolactone proved to be the drug of choice[7] In order to derive the maximum cidal action from the lactone ring structure, the drug and its reactions must be understood In addition, the reactions must be controlled, where necessary, if minimum alteration of labile components is to be preserved

Let me first point out some of the more important properties of BPL as a virucidal agent, then go on to show how these properties were employed to ob

under the 'Specially Purified' trade mark

tain maximum virucidal effect with minimal destruction of the physiological components necessary for clinical usefulness These properties were studied on viruses seeded in plasma[7,10] The properties that deserve emphasis are

(1) *The kinetic rate of BPL hydrolysis in relation to the rate of virus inactivation* FIGURES 1 and 2 show, respectively, the kinetic rate of BPL hydrolysis in plasma and the rate of virus inactivation at 37° and 4° C At either temperature and at points in between the virus is inactivated before the half life of the drug In numerous determinations the half life of BPL at 37° C varied from 24 to 32 minutes and at 4° C from 16 to 20 hours Virus inactivation with BPL to nondetectable quantities is attained in 15 min at 37° C and in 8 hours at 4° C If sterilization is to be conducted at 4° C, at least 4 days should be allowed for complete hydrolysis of the lactone before attempting to use the product, whereas at 37° C the hydrolysis is near completion at 2 hours and safe to use at 3 hours

(2) *Lack of virucidal activity of the products of BPL hydrolysis* BPL does not require a neutralizing agent to stop its reaction with proteins as it is a self limiting agent in this respect The products of degradation principally beta propionic acid and hydracrylic acid derivatives, possess no cidal activity in concentrations even higher than those resulting from BPL hydrolysis in the concentrations used for inactivating viruses[11] However, the acid products of hydrolysis must be converted to their sodium salts and adjusted to *p*H levels optimal for the stability of the biological product

(3) *Lack of pH influence on virus inactivation and the 'tailing' phenomenon* Because of the amphoteric nature of the drug during hydrolysis protein binding occurs over a wide range of *p*H levels without altering the rate of virus inactivation Although the bulk of the virus is inactivated by a relatively small concentration of BPL a disproportionate amount of drug is required to inactivate the residual and ultimate trace quantities of active virus This represents the tailing phenomenon[7]

(4) *Irreversibility of BPL inactivated viruses* The degree of virus inactivation cannot be reversed either by dilution prolonged storage or immediate neutralization with sodium thiosulfate[7] This compound will react with the residual BPL during hydrolysis and interfere with complete inactivation of MM virus when added at 5 and 15 min intervals as shown in FIGURE 3 samples 1 and 2 On the other hand, the addition of sodium thiosulfate at 30 min or after will not interfere with complete virus inactivation This also confirms the fact that drug virus binding is rapid stable and complete by the half life of BPL This property is particularly important in the sterilization of erythrocytes where the acid products of hydrolysis are more deleterious to the blood cells than is the primary reaction This point is discussed below

(5) *Effect of total protein content on the concentration of BPL required for virus inactivation to nondetectable quantities* When the virus titer is kept constant and the total plasma protein content varied the BPL concentration required [illegible]

% BPL | Log of Virus D n

100 — 10 9 — 10 8 — 10 7 — 10 6 — 10 5 — 50 — 10 4 — 10 3 — 10 3 — 10 1 — 0 — 0

4000 mg/L BPL
37° C.

1/2 L e
BPL

EEE

5 10 15 30 45 60 75

M I N U T E S

% BPL | Log of Virus D i n

100 — 10 9 — 10 8 — 10 7 — 10 6 — 50 — 10 5 — 10 4 — 10 3 — 10 2 — 0 1 — 0 — 0

4 000 mg/L BPL
4° C

1/2 Life
BPL

EEE

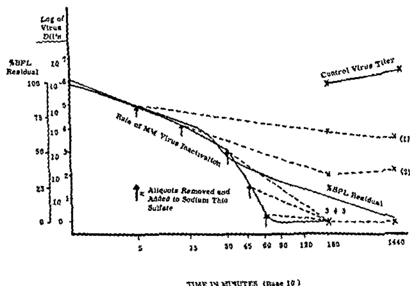
8 16 48 96 120

R S

F Rate of an ysis in 90 per
c t 4° C t s uantities be-
t rs w th s e half life
of s vari

were demonstrated with lymphocytic choriomeningitis (LCM) and MM strain of mouse encephalomyelitis viruses

(6) *Limitation of BPL protein binding* Since BPL is an excellent polymerizing agent, Basinski and Remp,[12] in our laboratory, undertook to determine the



[illegible]

Little Brown & Co Boston Mass

TABLE 1
SEDIMENTATION CONSTANTS OF HUMAN ALBUMIN*

BPL conc	Treated S_{20w}	Control S_{20w}
0 08M	4 73	4 53
0 14M	4 74	4 50
0 28M	4 72	4 55

* Maximum effect reached at $7\,4 \times 10^{-4}$ M during addition of BPL

effect of BPL on the sedimentation constants of separate fractions of human plasma The values were determined in a Spinco analytical ultracentrifuge at a speed of 50 740 rpm and for several concentrations of BPL

TABLE 1 summarizes the values for the three levels of BPL (0 08 M, 0 14 M, and 0 28 M) used in the treatment of albumen It is evident that the maximum effect on the albumen molecule has been reached at the $7\,4 \times 10^{-4}$ M BPL level, the excess BPL being hydrolyzed From Basinski's data[12] there was an

increase of 3000 in the molecular weight of treated albumen over that of un treated material There was an equivalent uptake of 42 moles of BPL per mole of albumen considerably less than the 180 or so titratable groups present in the protein Similar results were obtained with human globulin where there was an increase in molecular weight of about 2500 higher than the original un

of BPL per

ppears that

m as to the

amount of drug protein binding

(7) *Penetration of BPL into tissue and plasma clot* Failure to inactivate viruses completely is often ascribed to the size of particles in the infected ma terial In our studies on sterilizing tissue from autopsy material for human transplantation it became necessary to demonstrate the depth of BPL penetra tion for complete inactivation against viruses in tissue [13] This was best dem onstrated by sacrificing animals during the stage of viremia and assaying virus content in the tissue before and after treatment The thickness of the tissue slices varied from 1 to 9 mm It was found that 1 per cent BPL used for tissue sterilization was capable of inactivating LCM, MM, and EEE viruses from titratable quantities to nondetectable quantities if the compact tissue d d not exceed 3 mm in thickness and if the bone marrow slices did not exceed 6 mm in thickness that is a penetration depth of 1 5 and 3 mm, respectively In addition, splenic tissue from mice infected with AK_4 strain of mouse leukemia could be sterilized at a depth of 1 5 mm

More recently *E coli* T 3 phage enmeshed in plasma clots of 1 1 and 0 58 mm diameter and varying lengths* demonstrated that 0 25 per cent BPL in a 1 molar bicarbonate solution was capable of penetrating the plasma clot 0 5 mm in diameter (0 25 mm penetration) and reducing the virus activity to nonde tectable quantities whereas the thicker diameter of 1 0 mm was only partially penetrated reducing the virus activity from 7×10^8 particles to 2×10^4 parti cles Assay of the virus activity remaining in the plasma clot was made by digesting the clot with trypsin and releasing the active virus for suitable dilu tion studies The application of these properties of BPL has been most helpful in the treatment of plasma and other products

Thus far I have emphasized virus inactivation to nondetectable quantities

inactivate the hepatitis virus but still below the concentration which would alter the plasma proteins TABLE 2 gives the results of these studies (R Murray, personal communication) In the first human trial 50 ml of plasma was treated with 4000 mg /l of BPL, and 2 ml volumes were given intramuscu larly Of the 10 volunteers who received the treated plasma, none became ill, of the 5 volunteers who received nontreated plasma, 3 became ill In the sec

* Plasma clots of varying diameter are made in calibrated polyethylene tubing and then ejected

† Conducted in collaboration with Roderick Murray National Institutes of Health Public Health Service Bethesda, Md

ond volunteer study varying concentrations of BPL (2250 1250 and 700 mg /l) were tested against the same pool of hepatitis virus infected plasma The BPL concentration of 2250 mg /l seemed adequate to inactivate the hepatitis virus when 1 ml was administered intramuscularly Consequently, in the third human trial 3000 mg /l of BPL was chosen as an adequate concentration for absolute sterilization since it was more than the minimal effective virucidal concentration and below the concentration deleterious to the plasma components The results in the third trial clearly bring out the fact that trace quantities of virus in 600-ml volumes administered intravenously is a challenge different from a 1 ml volume administered intramuscularly Although most of the virus particles had been inactivated the persistent trace quantities manifested themselves in the larger transfusion volumes required for practical application This same point is emphasized in TABLE 3 which shows the results of a study made by different methods and volumes of assay of three of the viruses most resistant to BPL. Poliomyelitis virus for example showed that

TABLE 2
EFFECT OF BPL ON HEPATITIS VIRUS IN PLASMA AND EVALUATED IN MAN*

Year	Volume treated (ml)	BPL conc mg /L)	Volume and route of inoculation	Number of volunteers with hepatitis	Investigator
1952	50	4000	2 ml I M	0/10	J W Oliphant
1953	50 50 50	2250 1250 700	1 ml I M 1 ml I M 1 ml I M	0/10 1/10 3/5	R Murray
1954	7000	3000	600 ml I V	4/5	R Murray

* Unpublished data obtained from R Murray Division of Biological Standards National Institutes of Health

2000 mg l of BPL appears to be sterilizing when only 0 03 ml of the treated material was inoculated intracerebrally into mice but that 4000 mg /l of BPL was actually required when larger amounts of material were assayed in tissue culture by the more sensitive bulk test The same results were obtained with EEE virus and *E coli* T 3 phage Therefore the plasma problem became one of increasing the virucidal potency without increasing the alteration of the plasma components

Of the many combinations of chemicals and physicochemical virucides studied [7, 14, 17] BPL with ultraviolet (UV) irradiation offered the most promising results in overcoming our problem namely increasing virucidal potency without increasing alterations in the plasma components, toxicity or allergic reactions [19, 2]

The sharp contrast between BPL and UV used singly and in combination for virus inactivation has been reported It is often difficult to demonstrate the synergistic action of 2 virucidal agents by merely adding the log titer decrease in virus activity as small quantities of either agent are often capable of inactivating the bulk of the virus The effectiveness of the combination in our studies was made more impressive by measuring the concentrations re-

quired to shorten the tailing phenomenon and reduce virus activity to the base line zero One sixth of the drug concentration with one fourth the UV irradiation produced the same degree of virus inactivation as either agent used singly at greater concentrations Consequently, it was found that plasma could be treated with 3500 mg /l plus the standard UV irradiation intensity recommended for plasma by the National Institutes of Health[22] without serious alteration of the plasma components, particularly the coagulation factors

The essential amphoteric nature of BPL binding is indicated by the fact that electrophoretic studies show apparently small increases in mobility and small, if any, changes in the shape of the electrophoretic pattern TABLE 4 shows the effect of the combined treatment of BPL plus UV irradiation on

TABLE 3

CONCENTRATION OF BPL REQUIRED TO REDUCE VIRUS ACTIVITY TO NONDETECTABLE QUANTITIES VARIATIONS WITH METHOD OF ASSAY

Virus	10% Virus suspension in (Medium)	Virus titer (LD_{50})*	Method of assay	BPL conc (per cent)
Eastern equine encephalitis	Mouse brains in saline	9 0	0 03 ml I C in mice	0 2
	Chick embryo in saline	9 4	0 03 ml I C in mice	0 2
	Mouse brains in 90% plasma	8 5	0 03 ml I C in mice	0 3–0 4
	[illegible]	3 7	0 03 ml I C in mice	0 2
	[illegible]	6 4	0 1 ml /M K —cells (tube method)	0 3
	[illegible]	6 4	50 ml /M K —cells (bulk method)	0 4
	M K T C fluid in 90% calf serum	5 5	Bulk method	0 4
E coli T 3 phage	Filtrate in 6 mg %/ serum protein	9×10^8†	0 1 ml /plate	0 25
	Filtrate in 90% plasma	2×10^7	0 1 ml /plate	0 3
			5 0 ml /bottle	0 4

* Reciprocal of negative log of virus dilution
† Plaque count per milliliter of suspension

plasma components on 3 lots of plasma treated for clinical use The effect on the total proteins and electrophoresis protein distribution (in percentage) is given for each lot before treatment, and the effect of each step is shown in the procedure both with the drug alone and in combination There are relatively small changes in the plasma components in view of the virucidal potency of the combination Three times the drug concentration and 4 times the UV intensity were used than those necessary to reduce 1 of the more resistant viruses (*E coli* T 3 phage) to the base line zero

TABLE 5 shows the values for the coagulation factors of 2 of the same lots of plasma treated with BPL and UV singly and in combination The standard values for coagulation factors are given to compare the status of the plasma before and after treatment The total effect upon the coagulation components is reflected in the percentage of clottable fibrinogen These components are affected mostly by the acid products of hydrolysis rather than the protein

TABLE 4
EFFECT OF THE COMBINED TREATMENT* OF BPL PLUS UV IRRADIATION ON PLASMA COMPONENTS

ACD plasma treatment	Lot No	Total protein†	Paper electrophoresis protein distribution (percentage)				
			Alb	Alpha 1 glob	Alpha 2 glob	Beta glob	Gamma glob and fib nogen
None	No 9	5 3	45 5	4 7	8 4	15 7	25 6
	No 10	5 0	41 0	6 5	13 8	15 9	24 3
	No 11	4 9	43 3	5 4	10 0	15 8	25 4
BPL (3500 mg /L)	No 9	5 2	48 5	3 9	11 7	14 3	21 6
	No 10	4 8	42 5	5 6	9 2	17 4	25 1
	No 11	4 4	39 5	4 7	9 5	20 5	25 8
UV (Dill apparatus)	No 9	4 6	40 5	5 8	11 9	14 6	27 6
	No 10	5 1	39 2	6 9	13 4	17 2	23 3
	No 11	5 2	39 8	5 2	14 3	16 7	23 9
Combined BPL + UV	No 9	5 2	46 5	4 6	8 6	17 2	22 1
	No 10	4 7	45 8	6 1	9 4	14 9	23 8
	No 11	5 2	39 8	6 0	14 2	16 0	24 1

* BPL at 3500 mg /l and UV at 9 to 12 mw /sec /sq cm ((Dill apparatus)
† Protein grams per cent

TABLE 5
EFFECT OF BPL AND UV ON PLASMA COAGULATION COMPONENTS

ACD plasma	Lot number	Prothrombin time (sec)	Prothrombin 2 Stage (units/ml)	Plasma clotting time (sec)	Fibrinogen (mg %)
Untreated	Standard control	15	300	90 to 150	300 to 600
	9	22	108	168	284
	10	20	219	133	204
1 pH adjusted with 1 N NaOH from pH 6 8 to 7 6	9	42	108	340	226
	10	21	219	180	184
2 BPL treatment 3500 mg /l	9	85	49	300	0
pH not controlled during hydrolysis	10	66	102	435	0
3 UV treatment 8 to 12 mw /cm²/sec	9	26	108	364	212
	10	24	195	200	171
4 Combined treatment 3500 mg /l BPL plus UV	9	51	98	630	206
pH controlled during hydrolysis between 6 8 to 7 4	10	42	129	470	138

binding of BPL. Note how 3500 mg./l. of BPL in step 2 of the procedure will destroy the coagulation components when the pH is not controlled during BPL hydrolysis, whereas the clotting components are better preserved in step 4 of the same procedure when the pH is controlled during BPL hydrolysis. The prothrombin time and plasma clotting time were prolonged for each lot of plasma treated, and the percentage of clottable fibrinogen indicated some alteration, but not total destruction, of fibrinogen. Seventy-three per cent and 57 per cent of the original fibrinogen of lots Nos. 9 and 10, respectively, remained clottable. Six additional lots of treated plasma showed similar results. *The determinations for clottable fibrinogen seem more consistent than the fibrinogen values calculated from the electrophoretic patterns.* Moreover, for practical purposes it is perhaps more valuable to determine a physiological rather than a physical property.

To date 258 patients have received 691 transfusions of plasma treated by the combined method. Prior to this method of treatment, 414 patients had been treated with 1153 transfusions with plasma treated with BPL alone.[21] No toxic manifestations were noted in any of these patients that could be attributed to treatment of the plasma with BPL, nor have any cases of hepatitis been reported.

Since the combined method for treating plasma was proved the most satisfactory to date, the same principles were applied to the treatment of other biological material. Each product became an individual problem centered around the lability of the physiological components, the contaminating agents, and the necessary sterilizing concentration of BPL and UV used singly and in combination. The different products treated and the methods used are described.

Platelet factor 3. The combined use of BPL plus UV irradiation demon

[illegible]

xtracting platelet factor 3 from a bovine source for economic reasons and administering this product to thrombocytopenic patients.[24, 25] Sterilization studies were conducted with serum protein content equivalent to that found in the final platelet preparation, namely 6 mg. per cent.

TABLE 6 shows only those data pertinent to virus inactivation. The results shown are the effect of BPL and UV irradiation on *E. coli* T-3 phage seeded in 6 mg. per cent serum proteins when treated separately and in combination. The effectiveness of the virucidal agent can be followed in the third column under plaque counts. The original or nontreated material contained 9×10^8 virus particles per milliliter of suspension. The ultraviolet exposure of 22 μw./sec./sq. cm. did not sterilize the product. We also see that it took 2000 mg./l. of BPL to bring the plaque count to zero, whereas in the last 2 lines we

[illegible]

he physiological activity of the product; this is shown in TABLE 7. In the first column the amount of ultraviolet exposure indicated is obtained from one passage of the platelet material through the irradiation chamber. In the second

column the figures represent the concentration of BPL expressed in mg /l, and in the third column the amount of thrombin expressed in units per milliliter There is no significant alteration in the thrombin in the values shown except the last figure of 1400 units This figure falls just within the lower border of

Table 6
Effect of BPL Plus UV Irradiation on *E. Coli* (T3) Phage Seeded in 6 mg/per cent Serum Proteins in Normal Saline when Treated Separately and in Combination

UV exposure (mw /sec /cm.²)	BPL concentration (mg /L)	Plaque count (per ml)
None	None	9×10^4
22 ± 2	None	15×10^2
None	500	8×10^5
None	1000	13×10^3
None	1500	11
None	2000	0
None	2500	0
None	3000	0
22 ± 2	500	0
22 ± 2	1000	0

Table 7
Effect of BPL Plus UV Irradiation on Platelet Factor 3 (6 mg per cent of Protein N_2) when Treated Separately and in Combination

UV exposure (mw /sec /cm ²)	BPL concentration (mg /L)	Thrombin (units/ml)*
None	None	1690
22 ± 2	None	1700
None	500	1680
None	1000	1675
None	1500	1690
None	2000	1700
None	2500	1700
22 ± 2	500	1700
22 ± 2	1000	1700
22 ± 2	1500	1720
22 ± 2	2000	1580
22 ± 2	2500	1400

* Normal variation 10 per cent

normal variation for the method of determination In preliminary studies

which have been found with this product

The variety and frequency of the organisms isolated from 10 batches of platelet factor 3 prepared without aseptic technique and sterilized before clinical

use showed nothing unusual about the variety or frequency of the organisms found in a product prepared from bovine source A concentration of 1000 mg /l of BPL and the degree of ultraviolet irradiation employed sterilized the product from these organisms without significant alteration of the platelet material

Human erythrocytes Virus seeded whole blood can be inactivated with 4000 mg l of BPL However the acid products of hydrolysis cannot exceed 2500 mg l without hemolyzing the erythrocytes This has been reported in detail[7] briefly the present procedure consists of treating the erythrocytes and plasma separately A sterilizing concentration (4000 mg /l BPL) can be used

without producing hemolysis if the treatment is conducted at 4° C and the erythrocytes removed before the half life of the drug The application of the principles of treatment is demonstrated in FIGURE 4 It may be seen that when equal volumes of packed erythrocytes and BPL solution at a concentra

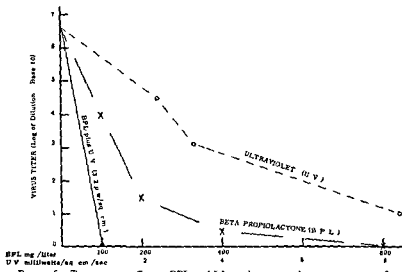
... ed to 4000 mg /l

Th s drug
zero before
erythrocytes

by the half life of the drug and resuspending them in an equal volume of citrate dextrose lactose solution the drug concentration is reduced further to approximately 2000 mg /l which is below the hemolytic concentration of the acid products of BPL hydrolysis

Having treated the erythrocytes with virucidal concentrations of BPL and having obtained satisfactory *in vitro* storage for 21 days, it remained to extend the work to *in vivo* survival studies in man To date, only 4 transfusions have

sterilization of plasma, warrant continued evaluation with improvement in removing the undegraded BPL by more thorough washing and less mechanical manipulation of the erythrocytes than is done in the treatment of these 4 transfusions Of all the virucides studied, BPL is the only agent thus far tested with which erythrocytes can be treated at virucidal concentrations without



producing hemolysis *in vitro* Moreover, the erythrocytes thus treated remain safe for intravenous administration in transfusion volumes

Inactivated virus vaccines With our results in plasma our attention was naturally turned toward inactivated virus vaccine studies [26, 27] Two viruses, EEE and CVS, were treated with BPL and UV singly and in combination FIGURE 5 brings out the tailing effect with each agent used singly, and the synergistic effect with the combination Similar results were obtained with both viruses Although these studies have been reported in detail elsewhere,[28] TABLE 8 is shown to emphasize the fact that the combined BPL plus UV treatment which inactivated the rabies virus to nondetectable quantities, maintained the same degree of antigenicity as the untreated control whereas concentrations of 800 mg /l and 1600 mg /l of BPL alone were required to obtain the same degree of virus inactivation, and the antigenic response was decreased,

particularly in the greater concentration The UV intensity was insufficient when used alone to reduce the virus activity to zero

The vaccines in these preparations were purposely prepared with relatively crude or coarse particles in the

are resistant to both agents is very small Whatever the mechanism of the combined action may be, the virucidal potency is definitely enhanced, and the antigenic component is better preserved What is equally important is the fact that BPL concentrations can be added in excess of that necessary to bring the virus activity to nondetectable quantities and thus ensure an added margin

TABLE 8*
ACTIVE IMMUNIZATION† IN MICE WITH RABIES (CVS) VIRUS
Vaccine Treated with BPL, UV, and Combined BPL plus UV

Vaccine preparation (ten per cent mouse brain suspensions)					Results of I C challenge (2 days after immunization)	
Virus inactivating agent	BPL conc mg /l	Ultraviolet irradiation: No lamps	Ultraviolet irradiation: mw /sec / sq cm	Infectivity LD_{50} titer log 10 (I C)	LD_{50} titer log 10	Resistance index
None	None	—	—	6 6	1 0	1 9 × 10^6
BPL, 37° C /2 hrs	800	None	—	0 0	1 5	6 3 × 10^4
	1600	None	—	0 0	3 3	1 0 × 10^3
UV irradiation	None	2	3 3	3 1	1 0	1 9 × 10^5
	None	3	8 4	1 0	1 3	1 0 × 10^4
Combined BPL plus UV	100	2	3 4	0 0	0 8	3 1 × 10^5
	200	2	3 8	0 0	1 0	1 9 × 10^6

* Reproduced from LoGrippo[28] with permission from the *Journal of Immunology*
† Method of Habel 0 25 ml I P every other day for 6 doses

of safety against infectivity and minimum alterations of the antigenic component

BPL[11, 12, 29, 32]

The usual practice of testing for bacterial and mycotic contamination over-

at the Henry Ford Hospital since 1953

Two hundred and sixty eight arterial transplants,[33] 117 bone transplants, and 15 cartilage transplants have been performed in man In no case was

there evidence of tissue failure due to inadequate sterilization, nor has there been any evidence of infection with fungi bacteria or viruses The results with cartilage although few in number [14] indicate that surface sterilization is adequate BPL sterilized tissues are dead as evidenced by tissue culture and are satisfactory only as prosthetic pieces and for temporary surgical procedures BPL-treated skin has shown no advantage over untreated skin as the rejection period for each is the same BPL treated corneal tissue in rabbits has shown that dead corneal tissue is unsatisfactory and that live corneal tissue is necessary for good results

Serum for tissue culture media Many of the difficulties encountered in tissue cell cultures can be attributed to toxic agents in the serum incorporated in tissue culture media [34 36] Although our filtration procedures* remove bacteria and fungi they permit pleuropneumonialike organisms viruses, and toxic substances to remain Such agents have been shown to be harmful to cell cultures and should be removed from serum used in growth media BPL is effective

TABLE 9
CELL LINES GROWN ON BETA PROPIOLACTONE (BPL) TREATED SERUM
Ten Per Cent Serum in the Growth Medium

Per cent BPL in treated serum	Cell line	Serum pool	Number of serial passages
0 35 + UV	HeLa	Calf	72
0 5 0 35 + UV	Detroit 6	Human Human	20 20 (total 40)
0 5	Chang liver	Human	11
0 5 0 35 + UV	HEp 2	Calf Calf	15 84 (total 99)

the serum does not exceed 10 per cent of the growth medium When serum is treated with 0 35 per cent BPL plus UV irradiation similar to the method described for treating plasma [17] 40 per cent of the serum can be added to the growth medium

BPL treated serum does not interfere with the susceptibility or the sensitivity of the cultures to virus agents *

* See Beta propiolactone—chemical procedure for the sterilization of serum for tissue culture media by B A LoGrippo C R Eidam and J L Truden To be published

Conclusion

In the sterilization of biological materials it is important that only the Specially Purified brand of BPL be used as this product contains at least 99 per cent of the lactone or active form of the drug whereas the commercial product commonly available contains approximately 82 per cent of the lactone form and 18 per cent impurities (acrylic acid acetic anhydride and polymers)

It is necessary to understand thoroughly the properties of BPL in order to apply them properly in the sterilization of biological materials as BPL improperly used can produce a wide variety of results Furthermore the variations in the concentrations of BPL necessary to sterilize biological materials vary with each product studied I cannot emphasize too strongly the fact that material sterilized with BPL with or without ultraviolet irradiation has shown no toxic or allergic manifestations when administered intravenously even in large transfusion volumes This is due to the fact that BPL is completely degraded by the time the material is used clinically and the products of hydrolysis are not toxic at the concentrations found This is understandable in view of the fact that beta hydroxypropionic acid and its salts which constitute

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CHEMICAL INACTIVATION OF FOOT AND MOUTH DISEASE VIRUS

O N Fellowes

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It was recognized at an early date that the virus was persistent in the animal during convalescence and in the excreta around the farm where the disease occurred The virus also survived in the carcass of animals dead of the infection[2-5] Early attempts were made to disinfect premises chemically and to dispose of dead animals in such a way that the farm or stockyard could be reoccupied by susceptible animals without ensuing reinfection[6] The earliest method used for the qualitative detection of the presence of virus one that is still used is to place a number of susceptible animals on the premises after a period of disinfection and quarantine Another means of detecting the virus became available to the laboratory with the discovery of the susceptibility of the guinea pig to FMDV[7] The quantitative estimation of virus by inoculating the tongues of susceptible cattle with dilutions of the infectious preparation was perfected by Henderson[8] Tissue culture of FMDV was introduced by Hecke[9] and further refined for quantitative determination of the virus by Sellers[10] and by Bachrach *et al*[11] The suckling mouse became a laboratory medium when its susceptibility was described by Skinner[12] Most of the work of laboratory detection and quantitative estimation of the virus has been accomplished by the inoculation of cattle guinea pigs, tissue cultures and suckling mice This selection of media and refined methods has been available only during the last five to ten years

The methods of testing for the effect of chemicals on the virus are influenced further by the comparative susceptibility of the various media used for the strains and by the types of FMDV employed Differences in susceptibility of different strains of animals to FMDV have been noted Tissue cultures based on single lines of cells may fall into groups of varying degrees of susceptibility to FMDV

A further complicating factor in the assessment of the effect of chemical environment on FMDV is the substrate in which or on which virus and chemical interact Most of the early work in this field was done with the virus in vesicular fluid or in lymph aspirated from the lesions formed by the virus on mucosal or epidermal surfaces In some instances filtration was employed to clarify such fluids after they had been diluted Tissue suspensions of lesion material from cattle tongues or guinea pig foot pads were used for some work At a later date tissue culture fluids containing FMDV were used as substrates to which chemicals were added Obviously as in earlier work with bacteria and viruses the amount of organic material present played a part in the inactivation In one instance the investigator wishing to conduct inactivation

under the worst condition added cattle feces and saliva to diluted filtered infectious vesicle fluid [13]

The background of information and observations just given is necessary to a better understanding of the research in this field The foregoing statement may appear naive, but it became worthy of mention after a survey of the literature on the subject had been made The chemical inactivation of FMDV will be examined from the arbitrary standpoint of the use of inorganic or organic preparations for such a purpose

Inorganic Chemicals as Inactivants

The use of sodium hydroxide to inactivate FMDV occurred very early in the history of FMDV [8,13,15] Minett[8,13] investigated the effect of sodium hydroxide on FMDV in diluted and filtered vesicle fluid Sodium hydroxide was added to the fluid to make the desired range of concentrations of alkal After the mixtures had stood for 3 hours at room temperatures samples were removed and inoculated intradermally on the plantar surfaces of the hind feet of guinea pigs The presence or absence of vesicles was noted and it was found that the concentration of alkali up to 1 20 000 destroyed the virus Olitsky *et al* [14] tested the effect of 0 25, 0 5 1, and 2 per cent sodium hydroxide on the virus in suspensions of guinea pig pads and in vesicular lymph The samples were taken from the mixtures of alkali and virus suspensions after contact of from 1 to 3 min In this study 1 and 2 per cent sodium hydroxide inactivated the virus in 1 min , as tested in guinea pig pads The same results were obtained when infected guinea pig pad suspensions were mixed with cattle urine manure or soil and treated with 2 per cent sodium hydroxide Olitsky and Boez[15] confirmed the foregoing experiments and extended the testing to include cattle inoculated by rubbing the treated virus into scarified mucous membranes of the mouth Again the various viral preparations were destroyed by exposure to 2 per cent sodium hydroxide for 1 min Blanc[16] added additional confirmation of this effect of sodium hydroxide on the virus

Sodium carbonate has been investigated by the British [8] and was found by them to be effective in a 4 per cent concentration that inactivated FMDV in 15 min at higher temperatures (60° C) but a longer time was necessary at a lower temperature The tests were performed with pieces of infected epithe

[illegible]

against a suspension of infected ground guinea pig pads and was able to inactivate the virus in 1 min

A concentration of 1 400 of sulfuric acid destroyed the virus in vesicular lymph that had been diluted and filtered At lower concentrations of 1 3000 to 1 25 000 there was incomplete inactivation However at 1 50 000 concentration of the acid complete inactivation of the virus occurred and continued

up to a 1 100 000 concentration These reactions were conducted at room temperature [13] A concentration of hydrochloric acid at 1 250 produced complete inactivation and at 1 400 to 1 5000 incomplete inactivation [13] Nitric acid was similar in effect to hydrochloric acid [13]

The relationship of inactivation of FMDV to pH and to acids and alkalis is an intriguing subject Minett[8, 13] feels that the fate of the virus when exposed to acid solutions does not appear to depend entirely upon the H ion concentration Thus the virus fails to live in certain buffer mixtures of pH 6 0 and

a pH of 3 4 does not inactivate the virus However a mixture of vesicle fluid and sulfuric acid at a 1 100 000 concentration to give a pH of 5 6 to 5 8 does destroy the virus To cover this type of result Olitsky and Boez[15] advanced the hypothesis that a coagulum formed with certain concentrations of chemical is dense enough to protect the virus Weaker concentrations of chemicals are thought to be lethal because the coagulum is not sufficiently dense to protect the virus finally the concentration of chemical is insufficient to produce a coagulum of the substrate or injure the virus

Pyl[7] had a unique explanation for the effect of acids or alkalis and the pH on FMDV Experimentally the virus in lymph from guinea pig vesicles is stable when exposed to pH 3 0 but is not stable when exposed to pH 6 0 to 6 5 The virus is most stable at pH 7 5 to 7 7 At a strongly alkaline pH such as 11 the virus is destroyed rapidly The active virus suspensions of FMDV held at low pH cannot be raised to the optimum pH level of 7 5 to 7 7 by the addition of alkali without losing infectivity However if the initial adjustment of the virus suspension is made to pH 11 0 the addition of acid to restore the pH to 7 5 will result in but little loss of virus activity This sequence of events is depicted in FIGURE 1 with FMDV and compared with vesicular stomatitis virus (VSV) The VSV does not resemble FMDV in its stability to pH The VSV was destroyed at pH 3 4 in 8 hours whereas at pH 4 0 to 10 7 the VSV survived exposure for 33 days The acid virus solutions that cannot be restored to the optimum pH level for stability without loss of infectivity are examples of the so-called realkalization effect

Since little of the pH or acid and alkali effects on the virus were performed with a substrate of infected bovine tongue epithelium such a series was started

posure period Samples were removed and tested for infectivity in cattle tissue cultures and mice at various time intervals up to 24 hours Some of these results are shown in TABLE 1 It may be noted here that all of these mixtures were adjusted to pH 7 4 from their initial pH of reaction In this instance realkalization seems not to have the effect Pyl found in his experiments

In comparison to the foregoing substrate Bachrach *et al* [8] used FMDV cultured in bovine kidney cells to test the effect of pH on viability of the virus

* Plum Island Disease Laboratory unpublished work

The clarified virus was diluted 1:10 in Michaelis Veronal acetate buffer to the various *pH* values used in the experiments. All samples taken at various time intervals were adjusted to *pH* 7.5 before inoculation into fresh tissue cultures. The results of these investigations appear in FIGURE 2. At *pH* 7 to 7.5 the virus was most stable. At *pHs* 8 and 9 the virus was inactivated 90 per cent at 3 and 1 weeks, respectively. At *pHs* 5 and 6 the virus was inactivated in minutes.

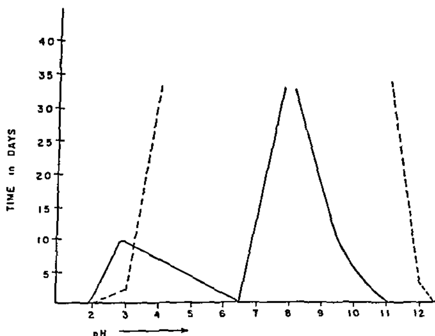


FIGURE 1. *pH* stability curves for FMDV and vesicular stomatitis virus in tissue suspension at 4° C. Reproduced with permission from *Zeitschrift für physikalische Chemie*.[17]

TABLE 1

INFECTIVITY TEST IN STEERS SHOWING EFFECT OF ACID AND ALKALI PREPARATIONS WITH DIFFERENT *p*H VALUES AT 37° C ON FMDV, TYPE A 119, IN TONGUE TISSUE SUSPENSIONS

*p*H	Chemical used	Hours exposed						
		2	4	6	8	10	12	18
13 0	Lye	—	+	+	+	—	—	—
13 0	Buffer	+	+	—	—	—	—	—
14 0	Lye	—	—	+	—	—	+	+
3 4	Phenol	+	+	+	—	—	—	—
3 4	Buffer	—	+	—	—	—	+	0
2 0	Buffer	0	0	0	—	—	—	—

Key: + primary and/or secondary lesions; —, not done; and 0, no visible lesion or positive serologic evidence of infection.

dried on cloth or sol d surface[21] Potassium permanganate sodium b sulfite sodium fluoride and chlorine do not have much v ral inactivating capacity in the presence of large amounts of organic matter[13 20]

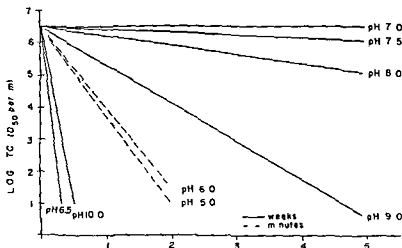


FIGURE 2 pH stab l ty at 4° C of type A 119 foot and mouth d sease virus grown in tissue culture Rep roduced with perm ssion from *Science*

Organic Chemicals as Inactivants

Citric acid was found to have an inactivating effect on FMDV in tissue sus pensions[5] A suspension of infected tongue epithelium from cattle was inacti vated in a 2 min exposure to 5 per cent citric acid as tested for infectivity in guinea p g pads An aerosol based on a concentration of 3 5 gm of citric acid per cubic meter of room space was created It inactivated dried virus placed in a container in the room after an exposure of 60 min Pieces of in fected whole tongue tissue were exposed to an aerosol of 3 5 gm of citr c acid per cubic meter of room space inactivation of the FMDV was complete in 6 hours[20]

Phenols and cresols are not too efficient in the inactivation of FMDV in vesicu lar lymph as tested in guinea pigs The complete inactivation of FMDV by phenols and cresols is limited to a concentration of 1 50 with the exception of ortho chlorophenol which had the desired effect in a concentration of 1 250[6 22] The presence of organic material cons derably delayed the in activation of the virus when phenols and cresols were used[14 2]

Chemotherapeutic agents of both organic and organic inorganic nature

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virucidal activity *in vitro* but not *in vivo*, with the possible exception of quinoline. The *in vivo* tests were conducted in guinea pigs.

Of the organic solvents, *n* butanol,[23] beta naphthol,[13] and chloroform[23] had little influence on infectivity. Chloroform, ether, and acetone added to dilutions of the virus did not harm the virus for a period of at least 4 days at room temperature.[24] Alcohol in concentrations of from 20 to 60 per cent has been added to vesicular lymph, to filtrates of vesicular lymph, and to centrifuged suspensions of tissue containing FMDV. Unfiltered virus will remain alive in the coagulum produced by the addition of 60 per cent alcohol to the vesicular lymph. The addition of 60 per cent alcohol to filtered vesicular lymph caused inactivation of the virus in 1 to 15 min. It has been pointed out that the coagulation is a function of *p*H. If 0.5 per cent NaOH is added to the alcohol to induce a *p*H of 8.2, the alcohol can then inactivate the virus. This theory was found to be true when 60 per cent alcohol with an approximate concentration of 1:5000 NaOH inactivated the virus contained in lymph or guinea pig pad suspensions in 1 min.[14,15] The infectivity tests were conducted in guinea pigs. Previously the problem of coagulation was encountered in the actions of acids and salts of heavy metals.

In early attempts to find disinfectants for FMDV, formalin was examined. It was noted that formalin was progressive in its action on the virus in relation to length of exposure. Formalin in a concentration of 1:50 was effective in inactivating FMDV in filtered vesicle fluid in 3 hours, and a concentration of 1:150 accomplished the same result in 24 hours. With infective tissue in suspension, a 1:20 dilution of formalin was required to inactivate the virus in 24 hours. A 1 per cent formalin solution would inactivate FMDV dried in tissue, such as guinea pig pads, in from 2 to 24 hours.[5,13] Early in the history of making vaccines against FMD, it had been shown that in preparations containing 0.15 per cent formalin held at *p*H 7.6 at 25° C for 96 hours the virus

bovine tongue tissue was used.[5] With the advent of culture of FMDV in living cells to high titer under controlled conditions,[10,11] it became possible to study the effect of formalin on the virus without the complications of a substrate containing much organic material. Wesslen and Dinter[27] studied the effect of formalin on virus in tissue culture at various *p*H values and temperatures of inactivation. In the experiments performed at 25° C by addition of 1:2000 formalin in a glycine buffer to produce *p*H 9.0, there was no significant difference in the rate of inactivation of any of the types of FMDV used. A composite of 6 different inactivations is shown in FIGURE 3. In similar experiments performed at 25° C by addition of formalin 1:2000 in glycine buffer to produce *p*H 7.0, there was little difference shown between types of FMDV in the rate of inactivation at this *p*H. A composite of the effect is indicated in

FIGURE 4 In these experiments of inactivation of virus at pH 9 0 25° C, 1 2000 formalin a remaining viral activity of 1000 LD_{50}/ml exists after 24-hour exposure time If 15 ml of a 1 per cent virus suspension is a vaccine dose, as is usually calculated with a stock virus of about 75 ID_{50}/ml, every vaccinated animal would receive about 150 ID_{50} of active virus [27] In the test just

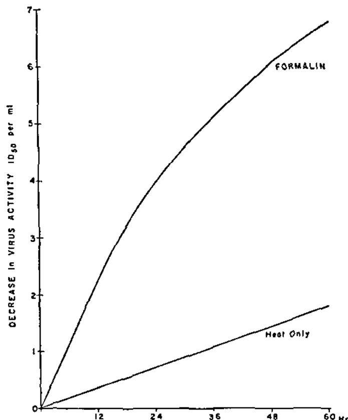


FIGURE 3 Inactivation of FMDV grown in tissue culture and exposed to 1 2000 formalin pH 9 25° C Reproduced with permission from *Archiv fur die gesamte Virusforschung* [27]

described no aluminum hydroxide was used but when it is a part of the vaccine, a viremia is sometimes produced in vaccinated cattle [28] Bachrach *et al* [15] studied a similar system of tissue culture virus treated with 0 009 per cent *formalin at 4° C held at pH 8 0 The virus content under these conditions* dropped about 1 log ID_{50}/ml /day as shown in FIGURE 5 It has been shown that the action of formalin on the virus was a chemical effect and not a pH effect and that optimum temperatures are necessary for a maximum effect [21]

In a recent study of 140 compounds for virucidal effect and toxicity, it was

noted that lactones, as a class, were nearly as active as the nitrogen and s mustards, but had less than one one-hundredth of the toxicity.[29] The pound beta propiolactone was found to be capable of inactivation of vi and bacteria at relatively low concentrations of the chemical in the presen organic material and with a minimum of modification of the antigenic acti

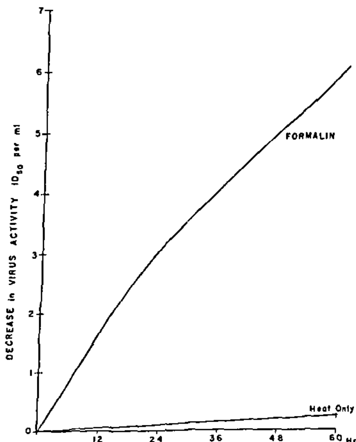


FIGURE 4 Inactivation of FMDV grown in tissue culture and exposed to 1 2000 formalin pH 7 25° C Reproduced with permission from *Archiv fur die gesamte Virusforschung*[27]

of the treated infectious agent[30] In studies performed at the Plum Island Animal Disease Laboratory (unpublished work) FMDV was readily inactivated by this chemical and retained most of its ability to act as an antigen in a complement fixation test with specific serum as shown in TABLE 2 Infectivity tests of the treated virus were performed in cattle

Another chemical capable of inactivating FMDV is ethylene oxide Since Phillips and Kaye[31] reviewed the effects of this compound on biological systems, the use of ethylene oxide has been applied to the inactivation of many viruses[32,33] Ethylene oxide can be used in either the gaseous or liquid form

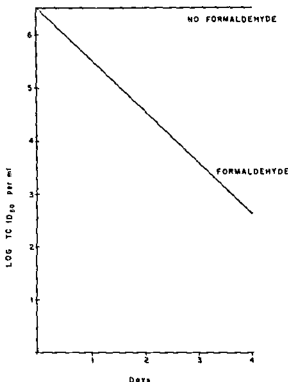


FIGURE 5 Inactivation of type A 119 FMDV grown in tissue culture and exposed to 0 009 per cent formalin at 4° C and pH 8 Reproduced with permission from *Science* [1]

TABLE 2
COMPLEMENT FIXING ABILITY OF TISSUE SUSPENSIONS CONTAINING FMDV TYPE A 119 TREATED OR UNTREATED WITH BETA PROPIOLACTONE

Antigen treatment	Dilution of antigen				Antigen control	
	1 10	1 20	1 40	1 80	Anticomplementary	Hemolytic
Virus control	4	4	4	3	0	4
Heated virus control	4	4	3	0	0	4
0 4% BPL 15 min 37° C	4	4	2	0	0	4
0 3% BPL 15 min 37° C	4	2	2	0	0	4
0 2% BPL 15 min 37° C	4	4	3	0	0	4
0 1% BPL 15 min 37° C *	4	4	4	0	0	4
Normal tongue tissue	0	0	0	0	0	4

Key 4 complete fixation of complement or no hemolysis 0 no fixation of complement or complete hemolysis and BPL beta propiolactone
* Contained living virus

and was first applied as a gas to FMDV by Savan[31] Cloth squares contaminated with FMDV were hung in a bottle and exposed to the gas phase of ethylene oxide at room temperature It was determined that the addition of 0 5 *ml* of liquid ethylene oxide per liter of container space inactivated the virus on the cloth in 30 min At about the same time a study on the effect of this chemical on FMDV in tissue suspension was initiated at the Plum Island Animal Disease Laboratory (unpublished work) The experiments were performed with the gaseous form of the compound in a special autoclave, and the results indicated that a concentration of 540 mg /l of airspace was necessary to inactivate the virus in tissue suspension in 4 hours, as shown in TABLE 3

TABLE 3
INFECTIVITY TESTS IN DIFFERENT MEDIA OF TISSUE SUSPENSIONS CONTAINING FMDV TYPE A 119, TREATED OR UNTREATED WITH ETHYLENE OXIDE GAS

Virus treatment	Medium used	Infectivity results	Complement fixing activity as antigen	Serum neutralization results
Virus control	Steer ID_{50}/ml	8 3†	+*	—
	Mice LD_{50}/ml	7 1		
	TC ID_{50}/ml	6 7		
Virus suspension held 4 hours at room temperature	Steer ID_{50}/ml	7 7	+	—
	Mice LD_{50}/ml	6 0		
	TC ID_{50}/ml	5 8		
Virus suspension (400 ml) treated with ETO for 4 hours at room temperature	*Steer*			
	SC—50 ml	0	+	+
	IM—50 ml	0		+
	IV—50 ml	0		0
	IDL—5 ml	0		0
	Mice LD_{50}/ml	0		
	TC ID_{50}/ml	0		

Key ETO, ethylene oxide, SC, subcutaneous, IM, intramuscular, IV, intravenous, *IDL, intradermolingual*
* Satisfactory in Traub Möhlmann complement fixation test at a dilution of 1 40
† Reciprocal of the logarithm

Volumes of suspension as large as 400 ml were tested It was observed that, when virus suspensions were dried on metal or gauze, even 18 hour exposure to a concentration of 540 mg /l of space in an autoclave did not inactivate the virus when moisture was absent, as indicated in TABLE 4 All tests were performed at atmospheric pressure Liquid ethylene oxide has been added to tissue suspensions of FMDV at 4° C and then reacted at 37° C Under these conditions a 2 per cent concentration of the compound in the suspension, heated for 1 hour at 37° C, will inactivate the virus and not greatly change its ability to act as an antigen in a complement fixation test with specific serum (TABLE 5) All infectivity tests were performed in cattle TABLE 6 shows the ability *of the chemically inactivated virus to act as an antigen in a specific complement* fixation test

Enzymes of various types have been used for the purpose of purification in the preparation of FMDV for further study, including electron microscopy

The virus present in a 10 per cent suspension of infected guinea pig foot pads lost its infectivity if 0 2 per cent of its volume of dry trypsin was added and the mixture incubated at 37° C for 24 hours at pH 7 8[25] A later study from the Plum Island Laboratory indicated that ficin, papain, and trypsin destroyed

TABLE 4
INFECTIVITY TESTS IN DIFFERENT MEDIA OF DRIED TISSUE SUSPENSIONS CONTAINING FMDV, TYPE A 119, TREATED OR UNTREATED WITH ETHYLENE OXIDE GAS

Virus treatment	Medium used	Infectivity results
Virus control	Steer ID_{50}/ml TC ID_{50}/ml.	7 8* 5 8
Virus dried 24 hours on gauze	Steer ID_{50}/ml TC ID_{50}/ml	5 2 3 9
Virus dried 24 hours on gauze and then treated with ETO for 18 hours	Steer IDL—5 ml IM—10 ml SC—10 ml TC ID_{50}/ml.	 2 positive 2 positive 1 positive, 1 NVL 0

Key ETO ethylene oxide, IDL, intradermolingual IM intramuscular, SC, subcutaneous NVL no visible lesion
* Reciprocal of the logarithm

TABLE 5
INFECTIVITY TESTS IN STEERS OF TISSUE SUSPENSIONS CONTAINING FMDV TYPE A 119 TREATED OR UNTREATED WITH LIQUID ETHYLENE OXIDE

Virus treatment (percent)	Route of inoculation	Amount of inoculum (ml.)	Infectivity results	Challenge ID_{50}/ml.
Virus control	IDL		7 9* ID_{50}/ml.	—
2 ETO, 1 hour, 37° C	IDL	5	0	8 0
2 ETO 1 hour 37° C	IV	50	0	8 6
2 ETO 1 hour 37° C	IM	50	0	6 7
2 ETO 1 hour 37° C	SC	50	0	7 2

Key IDL intradermolingual IV intravenous IM intramuscular SC subcutaneous, ETO ethylene oxide
* Reciprocal of the logarithm

TABLE 6
COMPLEMENT FIXING ABILITY OF TISSUE SUSPENSIONS CONTAINING FMDV, TYPE A 119, TREATED OR UNTREATED WITH LIQUID ETHYLENE OXIDE

Virus treatment as antigen	Dilutions of antigen				Antigen control	
	1 10	1 20	1 40	1 80	Anticomplementary	Hemolytic
Virus control	4	4	4	0	0	4
2 per cent ETO 1 hr 37° C	4	4	4	0	0	4

Key 4 complete fixation of complement or no hemolysis, 0, no fixation of complement complete hemolysis

and was first applied as a gas to FMDV by Savan[34] Cloth squares contaminated with FMDV were hung in a bottle and exposed to the gas phase of ethylene oxide at room temperature It was determined that the addition of 0 5 ml of liquid ethylene oxide per liter of container space inactivated the virus on the cloth in 30 min At about the same time a study on the effect of this chemical on FMDV in tissue suspension was initiated at the Plum Island Animal Disease Laboratory (unpublished work) The experiments were performed with the gaseous form of the compound in a special autoclave, and the results indicated that a concentration of 540 mg /l of air space was necessary to inactivate the virus in tissue suspension in 4 hours, as shown in TABLE 3

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Virus treatment	Medium used	Infectivity results	Complement fixing activity as antigen	Serum neutralization results
Virus control	Steer ID_{50}/ml	8 3†	+*	−
	Mice LD_{50}/ml.	7 1		
	TC ID_{50}/ml	6 7		
Virus suspension held 4 hours at room temperature	Steer ID_{50}/ml	7 7	+	−
	Mice LD_{50}/ml	6 0		
	TC ID_{50}/ml	5 8		
Virus suspension (400 ml) treated with ETO for 4 hours at room temperature	Steer			
	SC—50 ml	0	+	+
	IM—50 ml	0		+
	IV—50 ml	0		0
	IDL—5 ml	0		0
	Mice LD_{50}/ml	0		
	TC ID_{50}/ml	0		

Key ETO, ethylene oxide, SC, subcutaneous, IM, intramuscular, IV, intravenous, IDL, intradermolingual
* Satisfactory in Traub-Möhlmann complement fixation test at a dilution of 1 40
† Reciprocal of the logarithm

Volumes of suspension as large as 400 ml were tested It was observed that, when virus suspensions were dried on metal or gauze, even 18-hour exposure to a concentration of 540 mg /l of space in an autoclave did not inactivate the virus when moisture was absent, as indicated in TABLE 4 All tests were performed at atmospheric pressure Liquid ethylene oxide has been added to tissue suspensions of FMDV at 4° C and then reacted at 37° C Under these conditions a 2 per cent concentration of the compound in the suspension, heated for 1 hour at 37° C, will inactivate the virus and not greatly change its ability to act as an antigen in a complement fixation test with specific serum (TABLE 5) All infectivity tests were performed in cattle TABLE 6 shows the ability of the chemically inactivated virus to act as an antigen in a specific complement fixation test

Enzymes of various types have been used for the purpose of purification in the preparation of FMDV for further study, including electron microscopy

The virus present in a 10 per cent suspension of infected guinea pig foot pads lost its infectivity if 0 2 per cent of its volume of dry trypsin was added and the mixture incubated at 37° C for 24 hours at pH 7 8[35] A later study from the Plum Island Laboratory indicated that ficin papain and trypsin destroyed

TABLE 4

INFECTIVITY TESTS IN DIFFERENT MEDIA OF DRIED TISSUE SUSPENSIONS CONTAINING FMDV TYPE A 119 TREATED OR UNTREATED WITH ETHYLENE OXIDE GAS

Virus treatment	Medium used	Infectivity results
Virus control	Steer ID_{50}/ml TC ID_{50}/ml	7 8* 5 8
Virus dried 24 hours on gauze	Steer ID_{50}/ml TC ID_{50}/ml	5 2 3 9
Virus dried 24 hours on gauze and then treated with ETO for 18 hours	Steer IDL—5 ml IM—10 ml SC—10 ml TC ID_{50}/ml	 2 positive 2 positive 1 positive 1 NVL 0

Key ETO ethylene oxide IDL intradermolingual IM intramuscular SC subcutaneous, NVL no visible lesion
* Reciprocal of the logarithm

TABLE 5

INFECTIVITY TESTS IN STEERS OF TISSUE SUSPENSIONS CONTAINING FMDV TYPE A 119 TREATED OR UNTREATED WITH LIQUID ETHYLENE OXIDE

Virus treatment (percent)	Route of inoculation	Amount of inoculum (ml)	Infectivity results	Challenge ID_{50}/ml
Virus control	IDL		7 9* ID_{50}/ml	—
2 ETO 1 hour 37° C	IDL	5	0	8 0
2 ETO 1 hour 37° C	IV	50	0	8 6
2 ETO 1 hour 37° C	IM	50	0	6 7
2 ETO 1 hour 37° C	SC	50	0	7 2

Key IDL intradermolingual IV intravenous IM intramuscular SC subcutaneous ETO ethylene oxide
* Reciprocal of the logarithm

TABLE 6

COMPLEMENT FIXING ABILITY OF TISSUE SUSPENSIONS CONTAINING FMDV TYPE A 119 TREATED OR UNTREATED WITH LIQUID ETHYLENE OXIDE

Virus treatment as antigen	Dilutions of antigen				Antigen control	
	1 10	1 20	1 40	1 80	Anticomplementary	Hemolytic
Virus control	4	4	4	0	0	4
2 per cent ETO 1 hr 37° C	4	4	4	0	0	4

Key 4 complete fixation of complement or no hemolysis 0 no fixation of complement or complete hemolysis

the infectivity of FMDV when used to purify this agent from tissue culture fluids.[25] Infective ribonucleic acid preparations from FMDV became noninfective when treated with ribonuclease.[26]

The production of neutralizing antibodies by a viral antigen and the subsequent measurement of them are among the most specific and delicate tools that a virologist has at his command. The modification of FMDV by a chemical may be measured in this fashion. A test was devised at the Plum Island Animal Disease Laboratory to measure the response in the adult mouse to the administration of such modified antigens. Groups of adult mice, 25 to 30 days of age, received 2 doses of dilutions of the treated virus by the subcutaneous route at weekly intervals. One group of mice was carried as the control group. Three weeks after the administration of the first dose of vac-

Table 7

Illustration of a Test for Evaluating FMDV Vaccines in Adult Mice

Dilution of stock vaccines	Original vaccine dose/mouse (ml.)	Vaccinated mice		Cumulative total		Percentages surviving
		Living	Dead	Living	Dead	
1:20	0.005	5	5	8	5	61
1:80	0.00125	3	7	3	12	20
1:320	0.0003125	0	10	0	22	0

Calculations: $\frac{61 - 50}{61 - 20} = \frac{11}{41} = 0.270 \times 0.602 \text{ (log 4)} = 0.162$ correction factor

Log 0.005 = 7.699 − 10
Minus correction factor = 0.162

7.537 − 10

Antilog 7.537 − 10 = 0.0034 ml. of original 10 per cent vaccine protects against the LD_{50} in vaccinated mice.
Adult mouse controls: 38/39 dead.
Tissue culture controls, 3.75 LD_{50}/ml.
Suckling mouse controls, 5.5 LD_{50}/ml.

cine each vaccinated mouse received 5 mg. of cortisone and a dose of adult mouse-adapted FMDV, type A-119, which will kill 80 per cent of the controls receiving the same dose of virus and cortisone. The challenging virus was given by the intraperitoneal route. The volume, the concentration, or both, of treated virus preparation received by the mice, which protected 50 per cent of the test animals from death by the challenging virus, was calculated. This gave a numerical value with which to compare various preparations of treated virus similarly tested. It was possible, instead of challenging the treated mice with living virus, to bleed the groups of treated mice and perform a virus neutralization test in suckling mice with the various sera and get a different set of data. An example of the former type of test, which is less time consuming, is shown in TABLE 7.

The challenging research for the future in the chemical inactivation of FMDV is centered around the answers to three questions. *How does inactivation occur? Where in the chemical structure of the virus does it occur?* Is the same mech-

anism employed by each inactivant? It has been shown by Fraenkel Conrat[37] that formaldehyde is capable of reacting not only with protein, but also with the nucleic acid of viruses The discovery that the nucleic acid of tobacco

[illegible]

positive results[40]

Summary

It can be concluded that it is now possible to manipulate the chemical environment of FMDV so as to induce minimal changes or to destroy the virus completely The ability to conduct such studies has been improved through more refined methods of cultivating the virus and detecting the virus or its attributes

The research challenge for the future in the chemical inactivation of FMDV lies in the answers to the three questions How does inactivation occur? Where does it occur in the virus structure? Is the same mechanism utilized by each inactivant?

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26 RÖHRER H H MOHLMANN & G PYL 1950 Exptl Vet Med 1 1
27 WESSLEN T & Z DINTER 1957 Arch ges Virusforsch 7 394-402

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7 537 − 10

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Adult mouse controls 38/39 dead
Tissue culture controls, 3 75 LD_{50}/ml
Suckling mouse controls, 5 5 LD_{50}/ml

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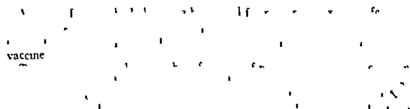
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A REVIEW OF THEORETICAL, EXPERIMENTAL AND PRACTICAL CONSIDERATIONS IN THE USE OF FORMALDEHYDE FOR THE INACTIVATION OF POLIOVIRUS*

Jonas E. Salk and John B. Gori
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tissue cultures and it had been shown that experimental vaccines could be prepared therefrom, the judgment that was then required concerned two simple questions: (1) How much formaldehyde should be used? (2) How long should

Preliminary Explorations

Before preparations of virus cultivated in tissue cultures were available, an effort was made to obtain a preliminary idea of the relationship between formalin (37 per cent formaldehyde) concentration and of the time required for inactivation, using preparations of virus contained in mouse CNS tissue.[2] The results of such an experiment are shown in FIGURE 1. These data suggested that, at the low temperature (4° C.) at which this experiment was carried out (which was assumed a priori to be the more satisfactory for maximal retention of antigenicity), high concentrations of formalin would be required to destroy infectivity within a time range that might, for practical reasons, be desirable. The lines describing the rate of decline of infectivity revealed a disconcerting degree of curvature when looked upon from the viewpoint of estimating time beyond which infectious virus would not reasonably be expected to be demonstrable in a laboratory test or when used in a potentially susceptible population.

Subsequently, and still early in the development of these investigations, when virus was propagated in roller tube cultures of monkey testicular tissue embedded in chicken plasma clots, assays for virus infectivity were, in some instances, more sensitive upon intracerebral inoculation of monkeys than in the then available testicular tissue cultures containing large quantities of animal serum. Because titration for residual infectivity were impracticable in monkeys, reliance for estimating treatment time and for approaching a safe level of destruction of infectivity was related to the point in time when infectivity had just disappeared as demonstrated in a sequence of samples taken at

* The studies referred to in this paper were supported in part by grants from the National Foundation, New York, N. Y.

appropriate intervals (FIGURE 2) In the later conduct of such experiments, formaldehyde action was interrupted by the addition of sodium bisulfite, and the undiluted fluid was then inoculated intracerebrally into monkeys When

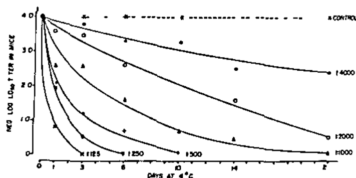
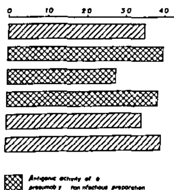


FIGURE 1 The effect of formalin upon the infectivity of the Lansing strain of Type II poliovirus different concentrations of formalin in 10 per cent mouse CNS suspension at 4° C*

Infectious Activity After Storage at 4°C for Days Indicated

COMPOSITE OF RESULTS OF TESTS IN MICE AND MONKEYS

CONC. OF FORMALIN	2	4	6	10	14	2
NONE	+	+	+	+	+	+
1:250	+	O	O	O	O	O
1:500	+	O	O	O	O	O
1:1000	+	+	+	+	O	O
1:2000	+	+	+	+	+	+
1:4000	+	+	+	+	+	+



high concentrations of formalin were used, necessitating large amounts of sodium bisulfite for neutralization, the resulting mixtures were toxic,[1] whereas mixtures consisting of lower concentrations of formaldehyde and correspondingly less sodium bisulfite were nontoxic Recognizing that an intracerebral test in monkeys would very likely be desirable, in addition to tests in tissue

ulture attention was then focused upon the effects of lower concentrations of ormaldehyde Because the reaction proceeds so slowly at refrigerator temperatures in the presence of lesser amounts of formaldehyde the new studies were carried out at incubator temperatures (36° to 37° C)

Inactivation of Virus at 37° C

As tissue culture methods improved it became possible to titrate for residual infectivity and to follow the course of formaldehyde treatment in samples removed at intervals (FIGURE 3) The course of the reaction observed in fluids available at the time of these early experiments at 36° or 37° C, revealed a linear regression with time at a rate that varied with formalin concentration but was independent of initial virus concentrations These observations sug

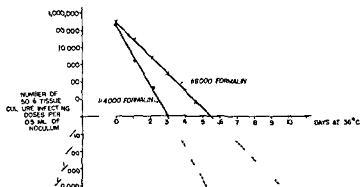


FIGURE 3 Rate of destruction of the infectivity of poliovirus in tissue culture fluid Virus was treated with formalin at 36° C and at pH 7 0

gested the possibility that simple extrapolation into the submeasurable range might provide a reliable basis for determining the time required for adequate treatment for destruction of infectivity[3] These data were more reassuring than those provided by reaction conditions at lower temperatures where curvilinearity was marked An additional theoretical advantage for the use of the higher temperature was in the greater prospect for retention of antigenicity (data on degree of retention of antigenicity are shown in FIGURES 4 to 9)[4] The reasoning here was as follows if conditions were selected in which curvilinearity was marked then prolongation of treatment time for adequate destruction of infectivity might result in excessive treatment of the major mass of virus inactivated early whereas under circumstances in which a rectilinear function is approximated such effects would be minimized

When these early observations were extended using virus suspensions prepared under different conditions and stored for longer or shorter periods of time it was observed in some instances that a more rapid drop in infectious titer occurred early after the addition of formalin subsequent to which linear regression was observed when the titer of residual virus activity was plotted against time

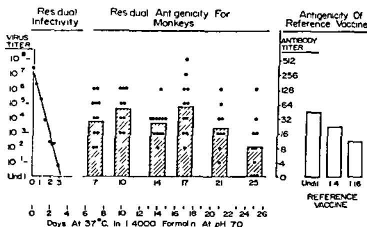


FIGURE 4 Dissociation of infectivity and antigenicity by 1:4000 formalin at 37° C (Mahoney strain of Type I poliovirus) The antigenic activity was tested by injection of 3 doses of 1 ml each, given 1 week apart, intramuscularly, to groups of monkeys The shaded columns indicate the geometric mean antibody titer, the solid symbols are the antibody titers in the individual monkeys [4]

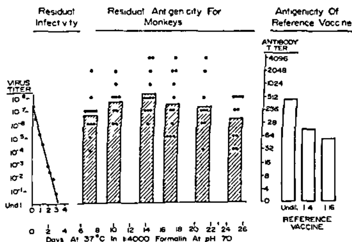


FIGURE 5 Dissociation of infectivity and antigenicity by 1:4000 formalin at 37° C

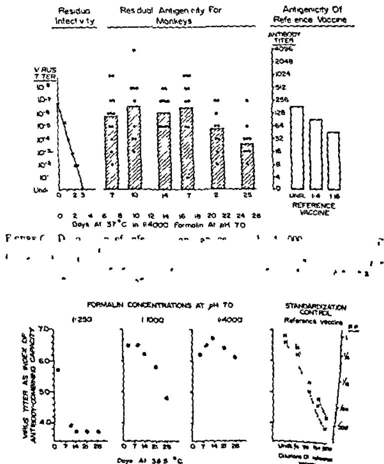


FIGURE 7 Rate of loss of antibody combining capacity as a function of for... ration (Mahoney strain of Type I poliovirus) R P is the relative potency ... with the reference vaccine [4]

In a memorandum prepared for manufacturers [6] attention wa... arly rapid decline for which no explanation was offered and ...

was made that the *zero time sample for* measuring the course of the reaction should be taken after the addition of formalin, or as soon thereafter as convenient

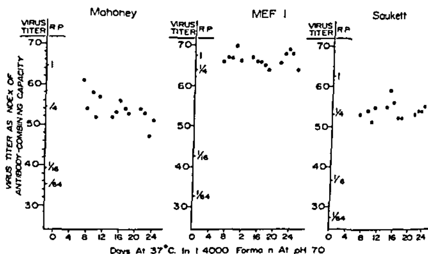


FIGURE 8 Antibody-combining capacity of samples removed at intervals under conditions of vaccine manufacture (experiment No 222) R P represents relative potency as compared with the reference standard as shown in the standardization control in FIGURE 7[4]

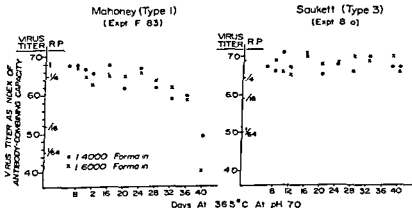


FIGURE 9 Degree of retention of antibody-combining capacity at intervals after the initiation of formalin treatment infectivity is no longer demonstrable after 3 or 4 days R P represents relative potency as compared with the reference standard as shown in the standardization in FIGURE 7[4]

On the assumption that the linear course would continue into the submeasurable range it was reasonable to expect that at a finite point in time virus particles in contact with formaldehyde in the particular reaction vessel would be expected to have become noninfectious (FIGURE 10) Accordingly, continuation of the reaction for at least such a calculated period and then for an addi

tional period, in consideration of nonmeasurable intangibles—such as relative sensitivity of the testing system—should result in destruction of infectivity with an added margin of safety. Under such circumstances, tests upon a sample of any size, including the sacrifice of the entire batch, should reveal no infectious virus unless a marked reduction in the rate of destruction of residual infectious virus occurred below the level of measurability. Such an effect could occur as a result of changes in temperature, or formaldehyde concentration, or if, by chance, virus particles were shielded from contact with formalin (FIGURE 11).

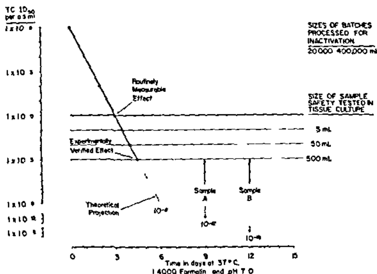


FIGURE 10. Combination of processing and control relationship between the measured rate of virus inactivation and the time of removal of samples for safety test. Reproduced by permission from *The American Journal of Public Health*.[7]

Vaccine Prepared for Field Trial

An experience such as this occurred in one of the earliest batches prepared by one of the two manufacturers who prepared vaccine used in the 1954 Field Trial. Because the intent of the filtration step was thought to have been the removal of bacteria or molds and not the removal of particulate matter that could interfere with the formaldehyde reaction, and because the fluid had been found to be bacteriologically sterile, a bacteria retaining filter was not used to remove the very fine particles, but simply a relatively coarse clarifying filter. The fluid, thus clarified, was treated with formalin, and virus was found in a sample removed at a point in time when it was to be expected that infectious activity had been reduced to a point below which it should no longer have been detectable. This was the only batch so treated, and all subsequent batches were subjected to Seitz filtration just before treatment with formalin. A summary of the results of tests for infectious virus in preparations of vaccine for

the 1954 Field Trial are shown in TABLE 1. As a matter of interest, the effect on filtration of infectious titers is shown in TABLE 2.

The trivalent vaccine pools prepared for use in the 1954 Field Trial were tested in

n

TABLE 3

keys, was [illegible] this was not as large a quantity as was later feasible and regarded to be more desirable. However, this limitation was com

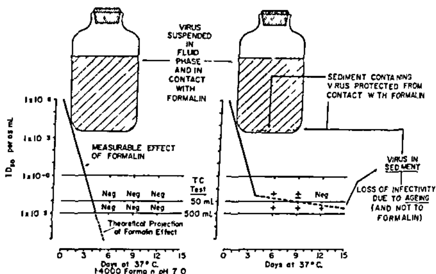


FIGURE 11. Effect of virus formaldehyde contact upon the rate of destruction of virus infectivity. Reproduced by permission from *The American Journal of Public Health*.[7]

TABLE 1
INACTIVATION OF VIRUS FOR 1954 FIELD TRIAL VACCINE[7]
(Experience of 2 Laboratories)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Original interpretation																				
Lab B	●	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Lab A	●	○	○	○	○	○	○	○	○	○	○	○	?							
* Revised interpretation																				
ab B	○*	○	○	○*	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
ab A	●	○	○	○	○	○	○	○	○	○	○	○	○*							

(Column heading: Sequence)

● = Positive or suspect
○ = Negative
* Based on re examination of CNS sections

pensated for by the requirement that it was necessary to prepare a consecutive series of lots, with negative tests for virus infectivity, before conduct of the Field Trial was approved This fact carried the implication that the vaccine preparation procedure itself was under test, as this would be reflected in consistency in effecting inactivation

If it is not unreasonable to regard all of the vaccine (except for the inadequately filtered lot referred to above) tested in tissue culture and in monkeys as representing a single sampling and to regard all of the vaccine produced for the Field Trial (with the exception noted) as a single batch, then a sampling of

TABLE 2

MEAN OF INFECTIOUS TITERS BEFORE AND AFTER FILTRATION OF TISSUE CULTURE FLUIDS CONTAINING EACH OF THE 3 TYPES OF POLIOMYELITIS VIRUS USED IN THE PREPARATION OF VACCINE FOR FIELD TESTS IN THE SUMMER OF 1954

Vaccine lots	Type 1 Mahoney		Type 2 MEF 1		Type 3 Saukett	
	Pre filt	Post filt	Pre filt	Post filt	Pre filt	Post filt
A 1 A 9	6 83	6 92	6 21	6 10	7 16	7 06
B 1 B 9	6 72	6 39	6 42	6 31	6 46	6 32
Average	6 77	6 71	6 32	6 21	6 81	6 69

Titers are expressed as the negative log of the 50 per cent infectious end point for tissue cultures using 0 5 ml inoculum in roller tubes prepared with trypsinized monkey kidney cell suspension

TABLE 3

ACCUMULATED CONSECUTIVE EXPERIENCE OF TWO LABORATORIES IN PREPARATION OF VACCINE FOR FIELD TRIAL USE

Lab	Lot nos	Volume of trivalent vaccine	Volume tested in T C and monkeys	No monkeys inoculated
A	12	1 400 000 ml	3312 ml	648
B	20	3 000 000 ml	5520 ml	1080
Total	32	4 400 000 ml	8832 ml	1728

8 8 l of vaccine may be said to have been subjected to test With a test volume of this size, the presence of one infectious unit of virus per liter, as measured in the test systems employed would have been detected, if present, with a probability that exceeded 99 9 per cent

Recognition of the importance of consistency as the prime consideration for safety is attested further by the fact that material prepared by a third manufacturer was excluded from consideration because of overt inconsistency Thus at the early stage when tissue culture methods were not sufficiently developed for testing more than small volumes, the requirement for a consecutive series of negative lots enhanced confidence in the adequacy of the procedure being used for destruction of infectivity

After the Field Trial

The development of a set of minimum requirements by the National Institutes of Health, Bethesda, Md, as a guide for control of vaccine manufacture was based in part upon the experience in preparing vaccine for the Field Trial[5] These requirements and the basis upon which they were constructed were re examined in April 1955 when a number of cases of paralytic polio, some of which were fatal and clearly attributable to the material injected, occurred among individuals given vaccine from one particular manufacturer The incongruity of this occurrence was striking when considered in the light of the Field Trial experience of the preceding year and in the light of concurrent field experience

TABLE 4
SIGNIFICANCE OF A CONSISTENT NEGATIVE RECORD IN PRODUCTION AND TESTING OF MONOVALENT STRAIN POOLS[7]
(Assuming Sensitive Testing and Representative Sampling)

Production sequence of strain pools (pool no)	Cumulative volume of strain pools produced (1 pool = 400 L) (ml.)	Cumulative volume tested in tissue culture (1000 ml /lot) (ml.)	Result of test for live virus	
			Example A*	Example B†
1	400,000	1,000	0	0
2	800,000	2,000	0	0
3	1,200 000	3,000	+	0
4	1,600 000	4,000	0	0
5	2 000 000	5,000	+	0
6	2,400,000	6,000	+	0
7	2 800 000	7,000	0	0
8	3,200 000	8,000	0	0
9	3,600 000	9,000	0	0
10	4 000 000	10,000	0	0
11	4 400,000	11,000	+	0
12	4,800,000	12,000	0	0

* Negative tests are less significant in proportion to the number of positives
† Negative tests are of increasing significance as cumulative experience is extended

with vaccines prepared by other manufacturers Without going into unnecessary detail, it is reasonable and plausible, from coded data contained in a report by the Public Health Service,[6] to deduce that vaccine, prepared under circum stances that did not consistently effect destruction of infectivity, had been submitted for release (TABLE 4) Thus, the basis upon which proceeding with the Field Trial had been approved and the basis upon which the minimal re quirements were constructed seemed to have been abandoned temporarily The implications (1) that the theoretical foundation upon which a formalinized vaccine was being prepared was unsound, (2) that the minimal requirements were inadequate, and (3) that new and more stringent test methods were neces sary, served to becloud the basic problem In addition, a host of theoretical explanations was brought forth, and the question of the dynamics of formaldehyde inactivation of poliovirus became a subject for lively discussion

Some investigators have taken issue with the explanation offered that the

inadequately inactivated vaccine was most probably the result of the protection from contact with formaldehyde of virus particles incorporated in cellular debris or chemical precipitates that were relatively impervious to penetration by formaldehyde. However, the adequacy of this explanation has been supported by subsequent experience. Earlier experience, already cited, as well as a priori considerations, had made this the most obvious explanation at the time the incident occurred.

Filtration for Effective Inactivation

In considering factors related to safety, attention had been drawn early to the possibility that tissue fragments, or isolated cells, or cell debris, might be present in the suspension and that these might contain entrapped or adsorbed

TABLE 5
EFFECT OF DEGREE OF FILTRATION ON EFFECTIVENESS OF DESTRUCTION OF VIRUS INFECTIVITY BY FORMALDEHYDE[4]

Extent of filtration through fritted glass filters*	Results of safety tests after treatment with 1:4000 formalin at 37° C for the following intervals of time: 5 days	9 days
No filtration	● ● ● ● ●	● ● ●
Coarse only	● ● ● ● ●	● ● ●
Coarse + medium	● ● ● ● ●	● ● ● ●
Coarse + medium + fine	● ● ● ● ●	○ ○
Coarse + medium + fine + ultrafine	○ ○ ○ ○ ○	○ ○ ○ ○ ○
Coarse + medium + fine + ultrafine + ultrafine	○ ○ ○ ○ ○	○ ○ ○ ○ ○

Each symbol refers to 100 ml sample.
Key: ● positive for virus; ○ negative after 3 weeks observation.
* 1 × 8" Candles in series. 17 Liters processed with 2.4 liters removed at each step for formalin inactivation.

virus that might not be as readily accessible to the formaldehyde as other virus particles that are free in suspension.[3] Although this is an obvious consideration, the failure to achieve removal of such particles may have been responsible also for the earlier unfortunate incidents with Venezuelan equine encephalomyelitis vaccine and may well have been responsible for failures heretofore in the development of procedures by which infectivity is destroyed to a degree where for all practical purposes, viable particles may be considered nonexistent. However, proof of the importance of filtration was first clearly demonstrated in connection with polio vaccine (TABLE 5).

It is now reasonable to presume, in retrospect, that the absence of viable [illegible]

filter failure, allowing a situation to exist in which virus was not brought into contact with formaldehyde, was indicated by the fact that exposure to 37° C for an additional 3 days after detection of traces of virus long after all should have been destroyed would frequently have no effect even though 6 $\log_{10}$ of infectivity loss were expected in the additional 3-day treatment period. Thus, it was clear that some gross deviation was in operation, the clue to which was provided by the incongruity between the data on the rate of inactivation and the results on safety test samples, suggesting that all virus particles in the reaction mixture in question had not necessarily come into contact with formaldehyde.[7]

A review of studies on the inactivation of viruses at this time affords an excellent opportunity for reflection upon the very large experience that has accumulated in the use of formalin for inactivation of poliovirus, and affords a further opportunity to re-evaluate and to bring into perspective the many ideas that were suggested to explain the episode of inoculation poliomyelitis that occurred in the spring of 1955.[4]

Dynamics of Formaldehyde Inactivation of Poliovirus

It was realized early that procedures customarily used for preparation of noninfectious vaccines would be inadequate for preparation of poliomyelitis virus vaccines, and especially for assuring the destruction of infectivity. A fundamental consideration for which understanding was required was the nature of the reaction between formaldehyde and virus. Mention has already been made of 2 relevant observations on the rate at which virus infectivity is destroyed, namely, the striking curvilinearity at 4° C and the essentially straight line function at 37° C.[2] Before embarking upon a discussion of such details it would be well to examine the results of the experience of a number of investigators.

Reference to the question of the dynamics of the reaction between formaldehyde and poliovirus was made in the following statement based upon experience with inactivation of virus at 36° C: "As with other antigens, interaction of formaldehyde with poliomyelitis virus, resulting in destruction of infectivity, occurs in accordance with the laws that govern a first-order chemical reaction. This means that if conditions are constant throughout the reaction period the rate, measured on a logarithmic scale, at which virus infectivity is destroyed, is constant."[3] Illustration of this observation is reproduced in FIGURE 12.

Open discussion of this question began with Gard's presentation at the Third International Poliomyelitis Conference, in Rome, Italy of data shown in FIGURES 13 and 14.[8] The contrast between these data and those illustrated in FIGURE 12 is as clear as is the similarity between the data shown in FIGURES 14 and 1. One point of view acknowledged the probable influence of different environmental conditions upon the reaction between formaldehyde and virus. Another point of view contended that the nature of the chemical process involved was not a one step, one bond phenomenon characteristic of a first-order reaction, nor even one of higher order, but rather "a gradual chemical transformation (tanning) associated with a gradual decrease in affinity to formaldehyde, *i.e.*, a gradually increasing resistance."[9]

If, in the course of inactivation, the continued decrease in the rate, as seen in FIGURE 5, is not as consistently observed as had been suggested, then an alternative interpretation to the "tanning" hypothesis would be admissible

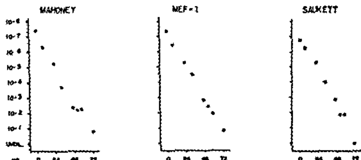


FIGURE 12 Rate of destruction of infectivity at 36.5° C in 1:4000 formalin at pH 7.0. The virus was grown at the University of Pittsburgh; filtration, formalin treatment and titration of samples were performed at the Wyeth Laboratories, Philadelphia, Pa.[4]

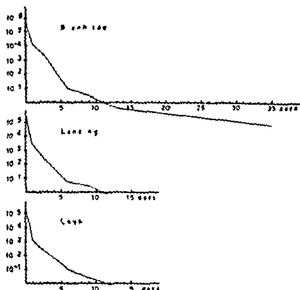


FIGURE 13 Typical formalin inactivation curves at a formalin concentration of 0.025 M in 0.1 M glycine at 0° C. Reproduced by permission from J. B. Lippincott Co.[9]

To indicate that a continual decrease in rate of inactivation in the course of the process does not always occur, the observations of Charney *et al.*[10] are reproduced in FIGURE 13. From this chart it is difficult to discern evidence for a continual decrease in rate of conversion to the noninfectious form; these authors state that ". . . the first-order inactivation kinetics which we have uni

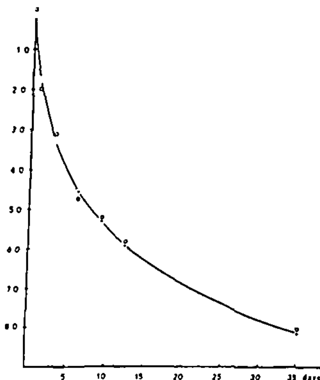


FIGURE 14 Formalin inactivation Ordinate, loss in infectivity, logarithmic scale, abscissa, time in days at 0° C The curve represents an exponential function The open circles represent mean values in 9 experiments Reproduced by permission from J B Lippincott Co [9]

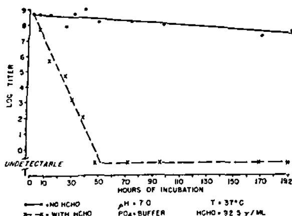


FIGURE 15 Formalin inactivation of pure Mahoney strain of Type I poliovirus Reproduced by permission from *Proceedings of the Society for Experimental Biology and Medicine* [10]

formly obtained are in agreement with the finding of [others]" Additionally, data reported by Lycke[11] and reproduced in FIGURE 16 indicate that a continual decrease in rate of reaction is not always discernible with time Still other illustrations (FIGURE 17) of exceptions to the suggested rule may be seen in the paper by Timm *et al*,[12] who had *not* observed a continued decrease, but rather a constant rate after the initial rapid drop That all of these experiences cannot be criticized for not having pursued the reaction to the practicable limit of observation is evidenced by more than an 8-$\log_{10}$ range observed by

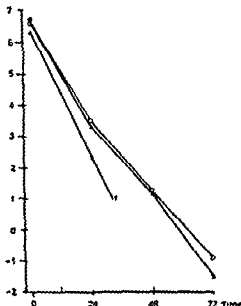


FIGURE 16 [illegible] 37° C [illegible]

Charney *et al*[10] and approximately the same range observed by Lycke[11] Studies from this laboratory over a slightly greater range had been made with the same findings and a very rigorous study by Bazeley (personal communication) in search for deviation in the submeasurable range has also revealed the same effects in tests of very large volumes of fluids

is observed, the rate of the reaction is independent of initial titer as Lycke *et al*[11a] (FIGURE 18) and others[12] (FIGURE 19) have also observed, and in this respect resembles a first-order reaction

Effect of Temperature of Reaction on Shape of Inactivation Curve

It has been pointed out that differences in shape were observed between inactivation rate curves at 0° to 4° C and 36° to 37° C[14] The difference can be related to effects mediated by temperature, in accordance with Maxwells law of distribution[15] as will be discussed later This becomes evident from a

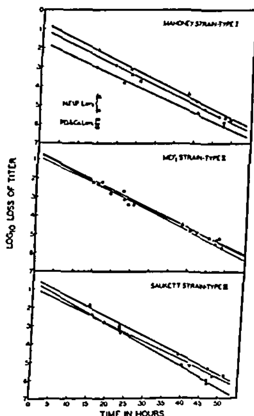


FIGURE 17 Formalin inactivation curves determined from the processing of 162 produc

derived from reports by Gard and his associates,[8, 9, 11, 13] who have reported many observations at 25° C and who have also reported findings at 0° C and 36 °C The curves in FIGURE 21 suggest an increase in curvilinearity with decreasing temperature of reaction, this effect can also be deduced from data reported by Lycke[11, 16] In further consideration of the effect of temperature,

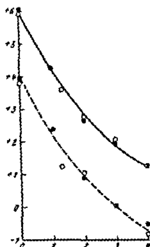


FIGURE 18. Inactivation of Type I poliovirus by treatment with 0.006 M formaldehyde

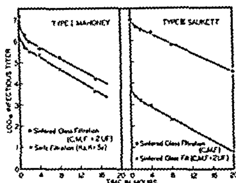


FIGURE 19. Effect of additional preliminary filtration of tissue culture fluid suspensions of the Mahoney and Saukett strains of poliovirus on subsequent inactivation by formalin. Reproduced by permission from *The Journal of Immunology*.[15]

Lycke has concluded that, when inactivation temperature is raised, the curvature from theoretical linearity (since he states that the basic reaction is a first order phenomenon) is corrected. This, he points out would be predicted from Gard's formula[*]

$$\frac{dY}{dt} = \frac{ab}{1 + bt} y$$

where an increase in parameter *a*, which would occur with an increase in temperature, would be reflected in a curve approaching linearity, Lycke went on to say that for this reason it might be desirable to inactivate at a temperature higher that 25° C However, on the basis of the fact that a temperature of 50° C, even in the absence of formaldehyde, destroys infectivity and antigenic

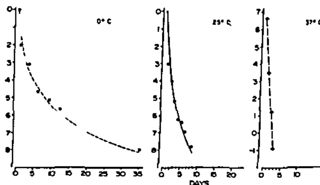


FIGURE 20 Effect of temperature on inactivation of poliovirus Redrawn from data in FIGURE 14 FIGURE 16[11] and from Gard[9]

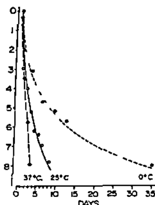


FIGURE 21 Composite on the same scale as the data in FIGURE 20

ity at the same rate, and in view of other considerations suggesting that a critical limit for the preservation of the antigen is as low as 25° C, Lycke[11] concludes that an increase in the inactivation temperature (from 25° C) is inadvisable It is not clear why an intermediate temperature in the region of 37° C might not be the solution to a more desirable temperature that is higher than 25° C and lower than 50° C

A further point of interest in relation to Gard's formula[9] when applied to data obtained at 25° C or 0° C, is that, as time advances, the curvature of the line gradually diminishes and then approaches linearity at a fixed slope The

relatively greater ease with which a margin of safety can be developed when

.. ..

in the region of 37° C or perhaps even slightly higher as compared to tempera tures of 25° C or 0° to 4° C

Let us now consider a concept of the nature of the formaldehyde virus reac tion that has been conceived to reconcile different observations made by differ ent investigators

A Concept of the Nature of the Reaction

A number of assumptions may be made regarding the nature of the virus particle with respect to its potential for reacting with formaldehyde If this potential depends on the virus particle alone it may be assumed (1) that all particles are exactly the same and that variability exists in the reactivity of the sites responsible for infectious activity or (2) that all particles are exactly the same without any variability in reactivity of the infectious sites or (3) that a population distribution of virus particles exists with respect to formalde hyde reactivity as would be expected for any biological system

If we consider that the third assumption reflects the nature of the virus par ticle with respect to formaldehyde reactivity then a first-order reaction would be described by the equation

$$l_{ln} \frac{I_0}{I} = Kt$$

(where I_0 = initial titer at time 0 I = actual titer at time (t) and the con stant K is a measure of affinity between formaldehyde and virus this constant is expressed in sec^{-1} and may be regarded as inversely related to the time re quired for reaction at the molecular level) The measure of time as expressed in FIGURE 22a is an expression of $1/K$ In this figure the vertical line repre sents a situation for a homogeneous population in which there is but a single value for $1/K$ the bell shaped curves represent a hypothetical normal distri bution of values for $1/K$ in a more or less inhomogeneous population where the degree of inhomogeneity would be expected to be reflected in the scatter of values for $1/K$ and this would then be reflected in the degree of spread of the distribution curve

The cumulative distribution of $1/K$ values for the 3 hypothetical possibilities is shown in FIGURE 22b where the vertical line that expresses a single value for $1/K$ reflects a homogeneous population and the slope and spread of the inter rupted lines reveal the character of the distributions of $1/K$ values for particles in populations of greater or lesser homogeneity In FIGURE 22c the curves described in FIGURE 22b are rearranged for K values rather than $1/K$ In

FIGURE 22d the straight line, in a semilogarithmic plot, represents the course of the reaction of an hypothetical homogeneous population, and the curved lines represent the course that would be followed by hypothetical populations of greater or lesser inhomogeneity, and the rates would reflect the variations suggested in FIGURE 22d. This schematic representation indicates that a pro-

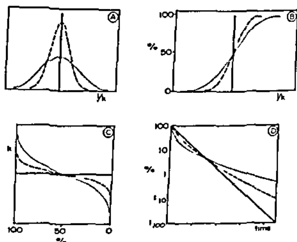


FIGURE 22. Population distribution of virus particles with respect to formaldehyde reactivity. (*See text.*)

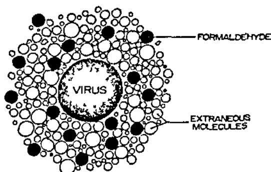


FIGURE 23. Schematic drawing of the physicochemical environment of a virus particle.

gressive increase in curvature of the inactivation course would be expected as inhomogeneity increases.

By way of further explanation it should be pointed out that the potential of the virus particles for reacting with formaldehyde would be expected to be influenced by the physicochemical environment in the reaction mixture and

acting with formaldehyde, and such modification could then be expected to be reflected in the course of inactivation

An extension of this hypothesis is suggested in FIGURE 24, which illustrates the effect that might be encountered when endogenous and/or exogenous factors result in population distributions, with respect to potential for reaction with formaldehyde, as extreme as here illustrated. As seen in FIGURE 24*a*, the interrupted line suggests that the major mass of virus particles is highly reactive and that smaller proportions are more slowly reactive; the converse is illustrated by the continuous line that suggests that the major mass is slowly reactive and that a small proportion is more highly reactive. FIGURE 24*b* shows the cumulative distribution of the value $1/K$ in 2 such populations, and FIGURE 24*c* shows the analogous distribution for the value K. It may be seen from

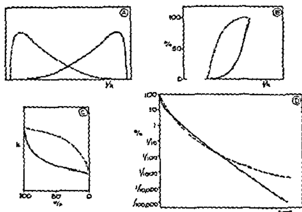


FIGURE 24. Effect that might be encountered when endogenous and/or exogenous factors result in extreme population distributions with respect to potential for reaction with formaldehyde. (*See text.*)

FIGURE 24*d* that the 2 different populations can be expected to exhibit different courses of inactivation. The interrupted line is reminiscent of the curves encountered by Gard,[9] whereas the solid line brings to mind the curves obtained by Timm *et al.*[14]

As a further extension of this analysis it is not difficult to see that under certain circumstances, such as might result in the narrowing of the distribution curves, reflecting increased homogeneity with respect to 1/K values, linear inactivation rate curves could be encountered such as reported by Charney[10] and such as have been observed in this and other laboratories. Charney has suggested that (1) the period of storage of virus-containing fluids, and (2) methods of purification that result in a lowering of the value for specific activity could account for inactivation curves of such character, as distinct from those described by Timm *et al.*[14] Thus, variability in the depth of the initial drop which Timm has reported, could be explained on the basis of factors suggested by Charney which could be expected to exert a greater

or lesser influence upon the width of distribution curves, which basically are of the type shown by the solid lines in FIGURE 24

It has been mentioned that factors affecting the degree of inhomogeneity of a virus population with respect to interaction with formaldehyde can be endogenous and/or exogenous *If inhomogeneity was due only to endogenous* factors (that is, to the nature of the virus alone) then under no circumstances should the inactivation curve be expected to be described by a straight line However, straight lines have, in fact, been observed, suggesting that under particular conditions the inhomogeneity of the virus population, if it exists is so small as to be imperceptible It must therefore be suspected that exogenous factors are the principal cause of inhomogeneity This surmise is supported by the distinct effect of temperature on the curvature of the inactivation course as demonstrated in FIGURE 21, similar effects resulting from variation in con

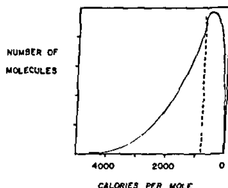


FIGURE 25 Redrawn from Bull [18] Maxwell distribution of translational energies of molecules at 25° C expressed in calories per mole The vertical line indicates the average energy per mole

centration of extraneous molecules in the reaction mixture, that is, glycine, have been reported

set
dis

in accordance with Maxwell's distribution law [16] This is illustrated in FIGURE 25, in which energies are expressed in calories per mole at 26° C The vertical line indicates the average energy per mole A similar distribution would be obtained using the value $1/K$ along the abscissa Maxwell's theory predicts that the distribution of energies is skewed toward the lower energies, the degree of skewing being more pronounced at lower temperatures As temperature is increased the curve approaches a Gaussian distribution, and the deviation from the mean is narrowed Thus, the hypothesis expressed by the solid lines in FIGURE 24, and the suggested influence of temperature on the course of inactivation are supported by considerations derived from the application of Maxwell's law of distribution

It would follow from this analysis that factors tending to increase inhomogeneity with respect to interaction between virus and formaldehyde, or that

interpose other molecular elements between free formaldehyde and the reaction sites on the virus particle, could alter the shape of the curve and prolong the interval over which curvature extends. Conversely, factors that tend to decrease inhomogeneity or that reduce the effect of extraneous elements would reduce curvilinearity and the interval over which this is evident. It is understandable, therefore, that under different conditions of temperature, nonviral impurities, and residual fraction of original specific activity, the shape of the inactivation curve would be different. Thus, under ideal conditions of inactivation and with an unbiased test for infectivity, the first-order characteristics of the reaction between formaldehyde and virus will express themselves as a linear function, under other conditions some other course will be described that appears to be expressed by empirically derived mathematical equations such as that suggested by Gard,[8] or other equations that are compatible with data of other investigators. This would not be proof that the reaction between poliovirus and formaldehyde is not a first-order reaction, nor that under practicably realizable circumstances the first-order nature of the reaction might not express itself.

Regardless of the precise conditions under which the ideal effects are approximated, the point of principal importance is that the choice of conditions for inactivation permits a selection that will tend in one direction or another. From general experience one of the conditions that would tend to favor approximation to the ideal, that is, a linear course expressing the first-order character of the chemical reaction, is a reaction temperature in the region of 37° C. That a first-order reaction in a biological system (which would be more correctly described as a pseudo first-order reaction) can be expected to exhibit deviations from linearity is axiomatic. It is therefore a technical matter as to whether conditions are chosen to minimize or exaggerate this tendency. It would seem, for the purpose at hand, that the reasonable objective would be to select conditions that would minimize this tendency.

It is perhaps fortunate that circumstances in this laboratory led to the selection of a temperature of 37° C and that the selection of another temperature by other investigators brought into clearer focus the indications of the existence of an optimal temperature for virus inactivation. It would appear, therefore, that if any general law can be deduced from these experiences, it is that, for viruses, the inactivation of which is basically a first-order reaction (as revealed by nondependence of inactivation rate upon initial titer) there are optimal conditions for effecting the most efficient destruction of infectivity, with the consequent possibility of greater retention of antigenic activity. It would follow that each virus requires its own optimal conditions to fulfill these objectives.

Discussion

It should be clear from the foregoing that the finding of viable virus in a reaction mixture at a point in time when none should be expected to be detected must be explained on some basis other than general theoretical consideration that might be deduced from studies at temperatures of 0° C or 25° C when the temperature employed under the circumstances in question was in the vicinity of 37° C. A negative test for viable virus should not be expected, either

uniformly or predictably, even in the presence of satisfactory inactivation rate data, unless the suspension is free of virus particles protected from contact with formaldehyde. In the absence of such protected particles, the course of inactivation at 37° C would be expected to proceed along an appropriately extrapolated course, and virus should then not be detectable after a predictable point in time, regardless of the size of the sample tested. That this has been accomplished when filtration has been adequate is attested to by the large quantity of virus converted into vaccine and safety tested* and used in the human population since the spring of 1955 without evidence of the presence of residual infectious virus.

Although the protected particle explanation seemed to be the most plausible one at the time when an explanation was needed, a number of other suggestions were made to explain inactivation irregularity. For example, it was suggested that the finding of live virus in a trivalent pool, when safety tests upon the three component monovalent pools were negative, could indicate the operation of some mechanism of recombination giving rise to a new viable unit. A simpler explanation of the observed facts would be that safety test samplings on the monovalent pools were negative because of sampling irregularity caused by the settling of protected viable particles in unrecognized sediment. Safety test samples removed from a trivalent mixture immediately after pooling could, under such circumstances, be expected more readily to yield live virus by virtue of the stirring of sediment that thereby had become accessible to the sampling pipette.

The idea that noninfectious virus might have an interfering effect in the test system, masking the presence of small quantities of infectious virus, was suggested by some to account for the finding of viable virus upon tests in monkeys when tests of larger volume in tissue culture were negative. The operation of an interference phenomenon would seem to be a less likely explanation than the fact that the particles containing viable virus may have been contained in insoluble material that remained insoluble in the safety test tissue culture medium of essentially the same composition as the vaccine, and that such insoluble material would be dissolved, and virus released, when deposited into the tissues of an animal. The possibility of the operation of an interference phenomenon was tested by performing infectivity titrations in tissue culture, comparing dilutions of virus in vaccine rather than normal diluting fluid, no difference was revealed between the two.

It has been reported that small amounts of infectious virus contained in fluids in which the interval is long past when infectivity should have been destroyed by formaldehyde make their appearance later in tissue culture than do traces of untreated virus. Under such circumstances it has been observed that, once infection is initiated, the subsequent sequence of events is equally rapid in cultures infected with treated or untreated virus. It has been dis

* [illegible] than 2 300 000

[illegible]

ml

proved that a genetically selected resistant variant is the cause of such an effect. The possibility must be considered, therefore, that the virus in such safety-test samples as result in delay may be contained in protected particles from which virus is eventually released, and that, when released, these particles come into contact with a susceptible cell, thereby initiating infection in the usual way. Additionally, it is not improbable that interaction of virus and formaldehyde would so alter the virus surface as to reduce the probability of effective contact between virus and cells for initiating infection, hence the delay.[17] This could occur at the step of virus-cell contact, at the point of permeation of the cell membrane, or even through reduced probability of contact between the infective site and the appropriate mechanism in the cell at which the mechanism for virus multiplication is initiated.

If the reaction proceeds in the expected manner and in the absence of factors masking the virus from contact with formalin, then such considerations as sensitivity of the detecting system become relatively less important, since the period of time that should be employed for inactivation should allow, in the [illegible]

negative test on a particular batch, the significance of a negative test on a sample of any size is greatly enhanced if it is one of a long series of negatives rather than one interspersed among positives.

A positive safety test is meaningful if data are also available on the rate of inactivation of virus in the particular batch, in the absence of such information it would not be po[illegible]

tected virus. A negative test is meaningful and significant if (1) adequate care in sampling is taken, if (2) a test system of adequate or known range of sensitivity is employed with observations made for a sufficiently long period of time to permit, the virus to be revealed and, most particularly, if (3) it is one of a series of negative tests on successive batches.

In continuing this discussion on the relationship between safety factors built into the method of vaccine preparation and the results of safety test samplings, it is desired to call attention to the work of Taylor *et al.*[11] who have given as one of the reasons for proposing the use of a combination of formalin and ultraviolet radiation[1] the relatively long periods of incubation (up to 18 days) required for "complete inactivation with formaldehyde alone." The conditions used by them (1:4000 formalin at 37° C.) should have accomplished this effect in less than one half this time. From the foregoing discussion it would seem reasonable to presume that virus particles not accessible to formaldehyde were present in the preparations with which they were dealing. Their studies have not excluded the possibility that better filtration or the use of other clarifying procedures that were necessary to permit satisfactory accessibility of ultraviolet radiation would not alone have provided for effective use of formaldehyde without ultraviolet radiation. These investigators have not established the fact that ultraviolet light is necessary for the effective use of form

aldehyde, nor have they demonstrated any advantage or benefit through the addition of the ultraviolet treatment The same may be said of the use of beta propiolactone in addition to formaldehyde

Another incident relating to safety factors built into the manufacturing process itself is revealed in connection with the statement that it was deemed inadvisable to pursue plans for vaccination in Sweden during the spring of 1955 in view of the finding of evidence of living virus in vaccine prepared in that country[19] It is important to note that the finding of live virus in the

nique produces an inactivation in roughly the same space of time as Salk's

no live virus

However

ot to have

included an adequate margin of safety, since a later report indicates that the time required for inactivation at 25° C with 1 2000 formalin is approximately 56 days

Timm *et al*,[12] have expressed the opinion that their data showed that first

inferences we draw from their data are quite the reverse of those they have

that the course of the reaction can be expected to be essentially linear ... over, their data indicate that a best fit line through more than 2 points, preferably 4 or more, would provide a reasonably good estimate for minimal treatment time, assuming that virus particles protected from contact with formalin were not present

have presented that the conditions

preparation of vac

ovide a reasonably

steep slope for the course of inactivation, moreover, one that approximates

then approaching the range of nondetectability Further

presumed to have gone to completion in 6 to 9 days, this can be ...

practicably since each additional day adds an appreciable increase in the safety margin A reaction that proceeds along a curvilinear course and is expected to approach termination in 8 weeks would require much longer treatment for equivalent additional safety margin because of the gradual slope, thus, weeks rather than days would be required Since antigenicity is well maintained during the additional days of treatment at 37° C beyond the point where treatment for destruction of infectivity is regarded as adequate, there would seem

to be no obvious advantage to a procedure employing a lower temperature in which the reaction is required to be carried out over a much longer period of time

Thus, the different effects observed by different investigators whose findings [illegible]

theoretically by Maxwell's distribution law but also experimentally, and, in practice the reaction at 37° C seems to proceed sufficiently closely to the pattern one would expect (if this were true) to make possible the establishment of a method for the preparation of vaccine reliably and consistently

A discussion of the use of formalin for the inactivation of polioviruses would not be complete without more than a passing reference to the incident of April 1955 already mentioned Unless some of the mystery surrounding this occurrence can now be cleared it may be said that little has been learned in the intervening years Although the present theoretical discussions have been interesting and, in many respects academically satisfying the fact cannot be escaped that this subject is also of more than casual interest in its practical implications We should be remiss if we did not inquire whether such an incident could occur again and if not why not Unless this question is approached with sound reasoning there will continue to hover over those responsible for vaccine manufacture an air of uncertainty along with fear and trepidation particularly when questions arise about improving or simplifying methods of manufacture including methods of testing

The first question that may logically be asked is whether the theoretical basis for vaccine manufacture is the same now as it was prior to the incident in question If the answer to this is in the affirmative then the next logical question would be whether sufficient knowledge was then available to have provided the basis for sound application If the answer to this is in the affirmative then it is logical to ask where the error was made

The impression exists that the vaccine released had met all requirements then in existence and that the government's minimum requirements were inadequate the fact that the requirements were modified after this incident is frequently used in support of this impression However if under the requirements as originally drawn up the existence of an unsatisfactory condition was evident is it possible that the modifications made in the minimum requirements after the incident may have beclouded the basic issue? Another way of testing the question would be to ask whether or not a similar incident could occur if merely the original version of the requirements were in force If the answer to this is in the negative that is to say that the original requirements had called for a reasonable number of safeguards then we must reach the conclusion that the incident was not due to the absence of warning signals but to the misinterpretation of their significance To select any one safety testing detail on the basis of which to explain away the incident is to avoid deliberately the basic concept that safety must be built into the production method itself '

This quotation is from a paper by Gard[9] who referring to specified volume testing, also said that " we can no longer rely on safety control tests of this type" In this same paper, Gard went on to say, "It is obvious that a well defined end product can be obtained only if inactivation runs a securely established and predictable course" The force of these statements, in part is in the agreement expressed with the "margin-of-safety" concept set forth originally and the requirement for "consistency" that was in the first set of minimum requirements and is still there through many revisions Although we may be at variance with Gard in how to accomplish these objectives we are in agreement with what must be done

Thus it would appear that the controlled manner in which poliomyelitis vaccine was to have been made represented a departure from the way in which killed virus vaccines had been prepared in the past Perhaps the admonition and the new knowledge available at the beginning did not take on meaning until their significance had been proved by disregard of detail Thus a critical examination rather than proving that the theoretical basis for polio vaccine manufacture and testing was unsound has proved precisely the reverse Now that the emotion related to the event itself is in the past, it may be seen that the additional safety testing that is performed on each batch, and that presently constitutes the largest part of the problem of manufacture, reflects most significantly satisfactory consistency This is the major safeguard that the minimum requirements, as originally written had specifically required when they called for Inactivation of virus by the use of an agent or method which has been demonstrated by the laboratory using the method to be consistently effective and reliable in inactivating a series of lots of poliomyelitis virus' As is customary in situations of this kind the details for so doing are left to the discretion of the individual laboratory It may not be irrelevant to say in this regard that the results of the application of principles must always remain the responsibility of those who accept the privilege of carrying out such principles

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THEORETICAL CONSIDERATIONS IN THE INACTIVATION OF VIRUSES BY CHEMICAL MEANS

Sven Gard

Karolinska Institutet Medical School Stockholm Sweden

Structure of Virus Particles

On the basis of chemical studies undertaken following the first successful purification experiments viruses generally were considered to represent giant nucleoprotein molecules This concept is no longer tenable In the virus particle a distinct architecture on a supramolecular level is recognizable apparently reflected in functional differentiation as well In principle, all viruses thus far studied consist of a core of nucleic acid (NA), or sometimes nucleoprotein, encased in a shell of non NA matter, chiefly protein

In several viruses the NA has been shown to carry all the genetic information needed for multiplication of the virus within its host cell (Gierer and Schramm 1956) Whether the same is true of all viruses must be left an open question for the time being Efforts to produce an active NA from myxoviruses have failed notoriously The reason for this is not known—it may be failure to introduce the NA into the cell or it may be that the protein plays a significant part in the intracellular virus synthesis At all events, it seems safe to assume that an active NA is a necessary condition for virus multiplication and that damage to the NA will incapacitate completely the virus as an infectious agent

The protein coat seems to have several important functions

(1) By its often remarkable resistance to chemical and other agents the protein coat serves to protect the NA, which is quite labile in the free state

(2) In some viruses a surface protein is the carrier of highly specific receptor mechanisms by which the entrance of the virus into the host cell is mediated It is also probable that the protein facilitates infection by viruses in which specific receptor mechanisms have not yet been demonstrated

(3) More hypothetical is the possibility that some protein constituent participates in the initiation of the intracellular process Recent observations indicate that the protein enclosed in the head of bacteriophage particles may have such a function (Spizizen 1957) The reconstitution of plant virus NA by addition of protein described by Fraenkel Conrat and Williams (1955), may point in the same direction

Apparently a true inactivation of a virus particle, that is a complete destruction of infectivity and capacity of multiplication must include an irreversible change in its NA Until recently inactivation of the NA was generally considered to be a one hit all or none phenomenon referable to essential sites in the molecule Most probably, however, the concept of particular chemical groups as carriers of the biological activity represents an oversimplification of the problem It seems more reasonable to regard activity as an expression of a complicated pattern of forces, determined by the structure of the molecule as a whole Any particular site is essential only as part of the pattern and any chemical alteration is important only insofar as it significantly modifies the field of forces For example, an amino group might be essential only because

by means of H bonds, it is being kept in a particular position relative *to adjacent chemical groups A substitution of one H atom might lead to* inactivation, not because maintenance of the intact NH_2 group is vital, but because the reaction may break an H bond, which in turn may bring the amino group out of line and distort the specific folding pattern of the molecule

With this reasoning inactivation as a two- or multiple hit phenomenon may easily be envisaged If, for example, two groups are linked by a double H bond, breakage of both bonds may be needed for disruption of the linkage Generally, the result will depend not only upon the point of attack, but also upon the nature of the chemical agent It is also conceivable that successive limited changes of the pattern of forces may lead to stepwise modifications of the biological activity before complete inactivation is achieved That, indeed,

nitrous acid

Kinetics of Inactivation

Inactivation of free NA has not been studied sufficiently as yet to permit any profitable discussion of the kinetics and nature of its reactions with various chemicals Experiments on native viruses present certain difficulties of interpretation, and conclusions should for that reason be drawn with caution These difficulties stem from two main sources the membrane effect and surface effects

(1) *The membrane effect* As already pointed out, the NA is encased in a protein cover In effect therefore it is separated from the surrounding medium by a semipermeable membrane A chemical agent can act upon the NA only if it is able in one way or another to penetrate this membrane Under these conditions the rate of inactivation obviously will depend entirely upon the rate of penetration which will more or less completely mask the kinetics of the reaction of the agent with NA

A chemical may be capable of penetration either because of its small molecular size or because of its capacity to break down the protein With the exception of certain enzymes the molecular size of which excludes the possibility of penetration no substances are sufficiently specific to react exclusively with NA and not at all with protein Therefore it may be considered as a general rule that an inactivating agent modifies the protein as well as the NA of the virus Whatever the type of the reaction changes in charge and hydration of the protein are to be expected which together with the structural alterations will affect its permeability In other words, in the course of the chemical treatment the rate of penetration of the protein hardly can be expected to remain constant and the rate of inactivation of the NA will vary accordingly

Theoretically a gradual breakdown of the protein should tend to increase the permeability of the membrane the consequence being acceleration of inactivation This phenomenon might be expected in acid or alkaline hydrolysis of viruses No sufficiently detailed data on the kinetics of these reactions to be available however

The opposite effect, a gradually increasing retardation of inactivation is to be expected in reactions with tanning or hardening agents causing fixation of the protein in the histological sense Formaldehyde is a typical representative of this class of substances By coverage of the amino groups it causes a considerable shift in the surface charge of the viruses, easily demonstrable as a reduction in *electrophoretic mobility* *More important in the present connection*, however, is the formation of new ring structures and bridges in the protein molecule and between adjacent molecules In this process the structure grows more dense, the membrane less permeable

As reported in 1954 at the Poliomyelitis Conference in Rome (Gard 1955) systematic studies of the effect of formaldehyde on poliovirus had shown the rate of inactivation to decrease in a regular fashion with the time of treatment

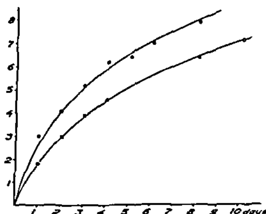


FIGURE 1 Inactivation of poliovirus by formaldehyde Log inactivation plotted against time

This was later amply confirmed, and a formula describing the reaction was proposed

$$\log y_0 - \log y = a \log (1 + bt) \qquad (1)$$

y_0 is the virus activity at time 0, y, virus activity at time t, and a and b, parameters

This formula has the following characteristics When logarithmic survival ratios are plotted against time, a continuously curving line is obtained (FIGURE 1) However when plotted against log time, the curve approaches a linear asymptote providing a possibility of extrapolation (FIGURE 2) The slope of the asymptote is given by the parameter a of the formula, its position by parameter b Finally when plotted against log $(1 + bt)$ the relationship is linear, and the fit of experimental data may be estimated easily (FIGURE 3)

The possible interpretation of the formula has been discussed in detail elsewhere (Gard, 1957) It may suffice to mention that of the 5 possible explanations, 4 could be excluded experimentally, leaving as the only remaining explanation the assumption that the formula describes the change in resistance

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*p*H affect both parameters

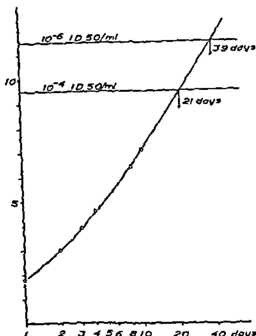


FIGURE 2 Inactivation of poliovirus by formaldehyde Log inactivation plotted against log time

For a further elucidation of the mechanisms of the *reaction*, it will be necessary to use methods other than purely virological ones Studies of incorporation of labeled formaldehyde, as reported by Meriwether and Rosenblum (1957) and now by Schaffer (*elsewhere in this monograph*), seem to offer excellent possibilities in this respect As another approach, the *measurement* of rates of penetration of chemicals through thin protein *membranes* would seem to provide means by which a direct study *of one of the principal* factors is possible

One more manifestation of the *membrane effect* might be expected At very low concentrations of the agent, diffusion toward the interior of the virus par-

* It might be added that an *inherent* heterogeneity of the virus, the hypothesis proposed by J E Salk elsewhere in this monograph, was *considered* as a theoretically possible explanation However, as no experimental data supporting this hypothesis *could be* produced, it was rejected

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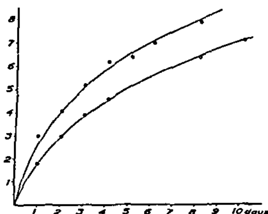


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of the virus to the action of formaldehyde in the course of treatment, that is, presumably the change in permeability of the protein membrane.* As the formula is empirical, the nature of its parameters is not immediately clear. It has been shown experimentally that a variation in formaldehyde concentration, other conditions remaining constant, causes a roughly proportionate change in parameter *b*, while *a* retains its numerical value. Reaction temperature and *p*H affect both parameters.

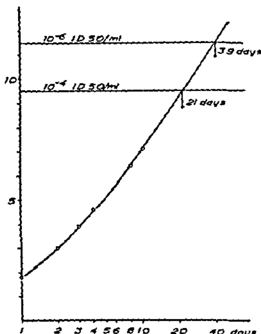


FIGURE 2. Inactivation of poliovirus by formaldehyde. Log inactivation plotted against log time.

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One more manifestation of the membrane effect might be expected. At very low concentrations of the agent, diffusion toward the interior of the virus par-

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ticle will be at a disadvantage in competition with the binding of the agent in the surface layers The diffusion pressure, already low, will suffer a further disproportionately great reduction The consequence would be a phenomenon similar to that observed in the action of disinfectants upon bacteria that is disproportionately weak effects at concentrations below a certain critical level. This seems in fact to be the case (FIGURE 4)

A compilation of data available in the literature has shown that most chemical agents seem to act on viruses in a manner similar to that of formaldehyde

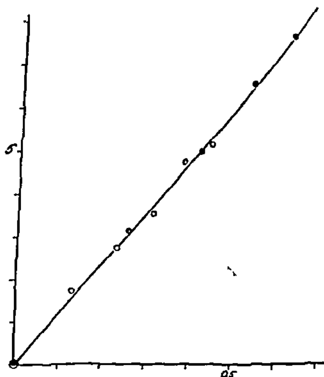


FIGURE 3 Inactivation of poliovirus by formaldehyde Log inactivation plotted against log $(1 + bt)$

that is the rate of inactivation tends to decrease with the time of treatment although not necessarily described by EQUATION 1 Some denaturing agents for example phenol and aniline, were reported to give inactivation curves of an extremely pronounced curvature, a rapid initial inactivation turning into a virtual standstill after comparatively short times (Lauffer and Stanley, 1943 Bawden and Pirie, 1940)

(2) *Surface effects* The infectivity of a virus particle is not exclusively a function of its reproductive capacity but also of its ability to enter the host cell and to undergo transformation from the extracellular resting state to vegetative virus For simplicity these qualities which in addition to the specific activity of the NA determine the infectivity of a virus particle, will be referred

to as avidity As already pointed out, at least some of these properties seem to reside in the protein or other non NA components of the particle There fore, chemical treatment of a virus might be expected to produce changes of its infectivity other than those strictly associated with inactivation of the NA

Bacteriophage, with its highly developed specific receptor mechanism, is a good illustration of this point It was recently reported by Spizizen (1957) that *Bacterium coli* phage T2 was inactivated by treatment with osmotic shock, presumably due to rupture of the protein cover and escape of the NA The

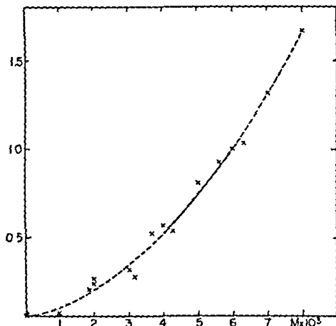


FIGURE 4 Inactivation of bacteriophage by formaldehyde Correlation between formaldehyde concentration (*abscissa*) and inactivation rates (*ordinate*)

naked protoplast of the host cell whose receptors are removed together with the cell membrane was resistant to infection by native virus, but could be in fected by the shock inactivated phage which interestingly enough also was able to infect the protoplasts of certain other bacteria possessing natural re sistance to native phage Shocked phage was not inactivated by DNAase, but by proteolytic enzymes Pure NA prepared by phenol treatment could infect neither protoplasts nor native bacteria Apparently this is a three-com ponent system (1) the NA representing the genome (2) the protein enclosed in the head of the phage particle and (3) the receptor apparatus the two halves of which are to be found in the tail protein of the phage and the membrane of the cell respectively Specificity of the receptors which allows the virus to enter its homologous host cell, apparently also may exclude it from a potential

heterologous host that in principle is able to sustain multiplication of the virus. Sidetracking of the receptor mechanism is possible, but only by sidetracking both ends of it. The interior head protein—as distinguished from the protein shell—seems to be essential for initiation of the intracellular process. Its precise function is not yet known; it may actually be an enzyme turning cytosine into hydroxymethylcytosine, a building block in the phage-DNA synthesis.

These observations serve to emphasize the relativity of the term infectivity. When measured in one test system, the manifestation of the structural change in the phage particle was a loss of infectivity, while the opposite effect was recorded in other systems. The infectivity spectrum was, in fact, completely reversed.

Another problem assuming particular importance in a discussion of surface changes is that of reversibility of reactions. The best known example of a completely reversible loss of infectivity again is to be found among the phages, namely the cofactor-dependent ones. However, sometimes inactivating agents in a more strict sense of the word also may cause a largely reversible loss of infectivity. Bacteriophage treated with formaldehyde seems to lose activity at an approximately constant rate, at least if activity tests are performed immediately at the time of sampling. If, however, the reaction is interrupted by addition of an excess of bisulfite, serum, amino acids, or other formaldehyde acceptors, and if the samples are kept at 37° C. at varying intervals before being assayed, a considerable gradual reactivation is demonstrable (Heicken and Spicher, 1956). FIGURE 5 is a schematic representation of this phenomenon. Both inactivation and reactivation follow approximately first-order courses, the latter reaction leading to a definite steady state. It is thus possible to draw two inactivation curves, one presumably recording surface changes, the other

less drastic but equally well demonstrable, provided that suitable methods are used. FIGURE 6 summarizes results of an experiment on poliovirus described by Böttiger *et al.* (1958). Virus was treated with formaldehyde, samples removed at intervals, and the reaction interrupted by addition of bisulfite. After determination of residual activities, concentrations were adjusted to equal ID_{50} values, and plates were seeded for plaque counts. As shown in FIGURE 6, untreated virus produced more than 50 per cent of the final count on the third day after inoculation and reached 100 per cent in 5 days. With virus exposed to formaldehyde for 24 hours, a delay of about 2 days in the appearance of plaques was observed. After 3 days of treatment the virus was exceedingly slow in producing lesions, the first plaques appearing only after 6 days' incubation; the final count was reached on the twelfth day. Apparently the possibility of maintaining the cultures in a viable condition sets a limit for the further exploration of this phenomenon. It should be added that, once started, the cytopathogenic changes seem to proceed at normal rates, irrespective of how the seed virus was treated; only the initiation of the process is delayed. Ap

Log remaining activity

Time

FIGURE 5 Inactivation of bacteriophage by formaldehyde. *Lower line* residual infectivity recorded immediately. *Upper line* residual infectivity recorded after reactivation. Arrows indicate extent of maximal reactivation.

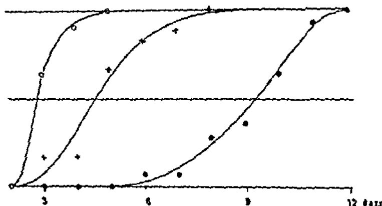


FIGURE 6 Time of appearance of plaques in tests with poliovirus. *From left to right* native virus, after exposure to formaldehyde for 24 hours and after exposure for 72 hours. Reproduced by permission from *Archiv für die gesamte Virusforschung*.

parently, treatment with formaldehyde causes a progressive change in the avidity of the still active, surviving *fraction of the virus, quite in line with the* fact that uptake of formaldehyde continues over very long periods of time (Schaffer, elsewhere in this monograph)

The significance of this observation is manifold In this connection only the technical side will be mentioned If, in the experiment just described, the final readings of the plates had been taken on the fifth day, the sample collected after 3 days' treatment would have failed to show demonstrable activity An inactivation curve based on such readings would have *had a much steeper slope* as well as a straighter course than one obtained by means of a more sen tive assay technique Actually, the two curves thus obtained may corre pond to the curves shown in FIGURE 5, one describing the surface changes, the other the reactions proceeding in the interior of the particle It seems not unlikely that some of the reported discrepancies in the results of formaldehyde inactiva tion of *poliovirus could be explained by* such technical differences

Inactivation in the Presence of Nonviral Organic Material

The particular problems encountered in inactivation of crude virus suspen sions as compared to purified material is of more practical than theoretical interest They may deserve mention in the present connection, however They are associated primarily with the reactions between the chemical agent and the nonviral organic material in the medium Formaldehyde again will be chosen as an example

Formaldehyde is a very versatile agent, capable of reacting with almost all kinds of organic groups—in the first place, with amino groups In aqueous media it forms methylene glycol, which is supposed to represent the reactive state In the presence of amino acids, as in a tissue culture fluid, it will enter into a large number of *different combinations, some of which are shown in* FIGURE 7 Most of these reactions are reversible, and the system therefore represents a very complicated state of equilibrium Furthermore, a number of the components are reactive and may be considered to possess a definite inactivating capacity, however, because of their larger molecular size, their ability to penetrate protein must be inferior to that of the free glycol Other compounds having no free hydroxyl groups are inactive but, due to the reversi bility of the reaction may yet serve as a formaldehyde reservoir, should the [illegible] of slowly proceeding irreversible

[illegible] a such

a medium and assay the concentration of reactive formaldehyde by any direct chemical methods For such purpo es a bio-assay seems the natural choice particularly a technique measuring directly the virus inactivating capacity, the quality on which information is sought Such a method, in whic [illegible] hylo-coccus phage is used as an indicator virus, has been devised ([illegible] r, 1959, in preparation) If due precautions are taken, this me [illegible] an accuracy of about 5 per cent in the concentration range of [illegible] total formaldehyde FIGURE 8 shows results of formaldehyd [illegible] technique, compared to data obta [illegible] a chemical meth

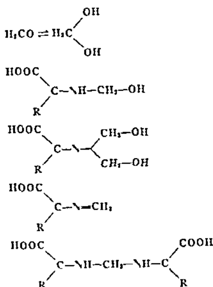


FIGURE 7 Formaldehyde in various combinations with amino acid

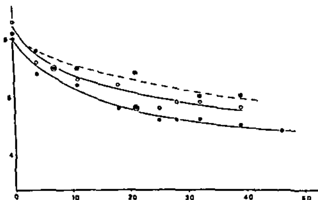


FIGURE 8 Assay of formaldehyde in tissue culture fluids *Abscissa* time in days at 25° C *Ordinates* formaldehyde concentration $\times 10^{-3}$ M Chemical determination of free formaldehyde ⊞ unfiltered + filtered fluid Bio-assay of active formaldehyde ● unfiltered ○ filtered fluid

between the 2 fluid , the unfiltered portion suffering the greatest reduct on in reactivity

It should be pointed out first that this relatively slow di appearance of reactive formaldehyde cannot account for more than a small fraction of the retardation of inactivation referred to as the membrane effect It is however of considerable practical importance as a disturbing factor in experimental work and in the production of inactivated virus vaccines The formaldehyde-b nding capacity varies from one batch of tissue culture to the next and therefore will affect the reproducibility of results Needless to say, uniform and reproducible results cannot be obtained unless the conditions of the reaction are standardized and kept constant

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INACTIVATION OF CONCENTRATED PURIFIED POLIOVIRUS SUSPENSIONS

Jesse Charney, W P M Fischer, Jerome F Sagin, Alfred A Tytell
Merck Sharp & Dohme Research Laboratories West Point Pa

This paper describes some of our studies on the inactivation with formaldehyde of highly purified, concentrated suspensions of poliovirus

Without pretending to offer a definitive discussion of the kinetics of poliovirus inactivation, we outline the considerations that led us to undertake these studies These are suggested in FIGURE 1, in which the following possibilities are indicated

(1) With respect to viral inactivation under chosen conditions, does there exist a nonhomogeneous population of virus such that a detectable fraction of virus suffers a relatively rapid loss of infectivity with respect to a more stable surviving fraction (*ac* or *bc*)?

(2) Apart from that portion of the inactivation curve near the origin does the remainder of the curve exhibit first-order (or more properly, pseudo first order) kinetics (*cde* or *cdf*)?

(3) Is extrapolation justified, what does the curve look like below the line (below the limit of detectability of the test) (*eh* or *eg*)?

This question is open to a number of interpretations

(1) Assuming a population of uniform infective particles not all of which give rise to an observable result in the test chosen (that is the test is insensitive) no problem arises Since the particles are assumed to be uniform the kinetics exhibited by those successfully detected are representative

(2) The hypothesis that a type of infective particle exists that is not detectable by the assay selected is of course not tested by the experiment a number of different means of measuring of infectivity may increase confidence

(3) The most straightforward interpretation of the nature of the curve below the line is simply Does a minute population exist different in kind and less quickly inactivated than the bulk of infective virus? A sufficient number of such particles would exhibit a reduced rate of inactivation at the end of the curve, but the required number may not be present in the sample chosen

To answer this question, testing of a concentrated preparation is indicated Moreover, to avoid a great increase in the concentration of extraneous substances possibly capable of influencing the kinetics of inactivation purification also is required

Previously we have published[1] studies of the inactivation with formaldehyde of concentrated, purified poliovirus suspensions prepared by a modification of the methods of Schwerdt and Schaffer[2,3] using as our starting material aged poliovirus-infected tissue culture filtrates (TCF) No departure from first-order kinetics was observed throughout the course of inactivation In FIGURES 2 3 and 4 we report some of our studies with poliovirus prepared from relatively fresh TCFs by a recently developed method (as yet undescribed in print) that yielded essentially pure virus as determined by spectrophotometry and nitrogen analysis In the case of the Type I (Parker) preparation ultracentrifugal sed

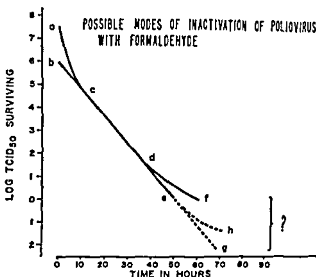


FIGURE 1 Theoretically possible modes of inactivation of poliovirus The possibility of a curved portion (*ac*) at the initiation of inactivation and a curved portion (*df*) at the end of inactivation are shown in distinction to complete first-order kinetics (*bcde*) The dotted lines *eh* and *eg* illustrate the possible continuation of the curve with more concentrated preparations

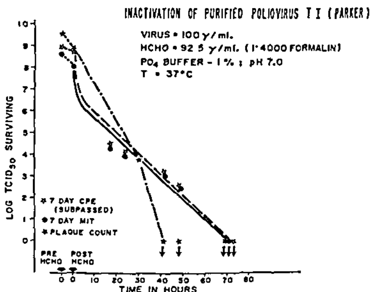
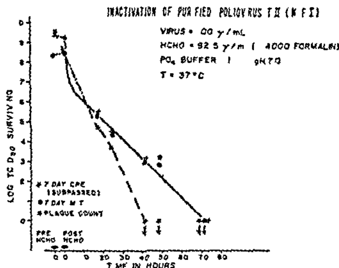
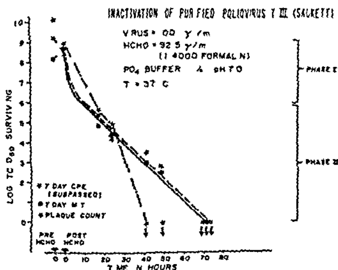


FIGURE 2 Experimental results with purified, concentrated poliovirus (Type I, Parker) The region from $\log_{10} = 0$ to $\log_{10} = 2$ usually is not seen with crude virus-containing fluids Arrows indicate undetectable infectivity in undiluted preparations



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effect (CPE) in roller tubes, metabolic inhibition (color) test (MIT) and plaque count Near the end point of infectivity, negative tubes (frozen and thawed 3 times) were subpassed The subpassages were held for observation for 18 to 29 days with periodic refeeding Subpassage did not increase appreciably the over all sensitivity

From the data given in FIGURES 2, 3, and 4, a number of conclusions may be drawn

(1) The CPE assay is very slightly more sensitive than the color test Plaque assay is at least equally sensitive with respect to untreated virus but, under the conditions of the test, it fails to detect formaldehyde-exposed virus that is infective in the CPE and MIT tests Schultz *et al*[4] have pointed out that exposure of poliovirus to formaldehyde produces a type of damage that results in an increased delay in the appearance of plaques This delay becomes progressively greater with extended treatment If a virus-cell adsorption effect is involved as Schultz suggests, the desensitization of the plaque assay as compared with the CPE and MIT tests may be explained The conditions of virus cell adsorption are very different in the latter test systems Considering this effect it is obvious that if under other conditions of plaque-count assay a curvilinear course of inactivation was exhibited near the end point little fundamental significance could be attributed to it

(2) Our data indicate that a fraction of the population suffers a relatively rapid loss of infectivity (Phase I) with respect to a more stable surviving fraction (Phase II) This phenomenon has been pointed out by Timm *et al*[5] I should add that the magnitude of this phenomenon depends upon the previous history of the preparation In TCF stored for long period , this loss of unstable infectivity seems already to have occurred

Failure to distinguish between the two phases of inactivation or conducting the inactivation under such conditions that the first phase is protracted with respect to the second may lead to the conclusion that the course of inactivation departs throughout from first-order kinetics This effect may explain the differences in interpretation of the inactivation kinetics of poliovirus in various stud es

(3) Under the specific conditions employed in this study, first-order kinetics were exhibited after the initial rapid inactivation phase was completed

(4) Inactivation of virus at 100 μg/ml (one hundredfold concentrated with respect to ordinary TCF) sensitizes the test by a factor of 100 to the presence of a hypothetical minute population of less rapidly inactivated virus particles No evidence of such a population appears

We conclude that, with due regard to the specific conditions employed and ... kinetics the inactivation ... which a pred c ... ntrol in vaccine

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Part III Physical Inactivation of Viruses

THEORY OF THE PHYSICAL MEANS OF THE INACTIVATION OF VIRUSES

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Introduction

The physical methods most commonly employed for the inactivation of microorganisms are thermal action ultraviolet light ionizing radiation pressure and sonic radiation These methods do not operate by any means in the same way and rather than attempt to group them in any pattern by their physical characteristics it is much more rewarding to consider first the features of a virus and how these are related to the effect of various physical agents upon them Accordingly, I shall discuss the basic concepts of the way in which a virus functions in such a manner as to facilitate the description of physical action upon them

Viral Nature and Function

Perhaps the best way to consider these two very important subjects is to examine three schematic diagrams of animal bacterial and plant and insect viruses (FIGURE 1) Such diagrams while they represent somewhat the present knowledge about viruses are not intended to be exact but they are intended to focus attention on the six aspects of virus action that could be influenced by physical agents

— ... esented in FIGURE 1 (*left*) It is shown ... l onto a protein ... preservat e ... protein and the coiled nucleic acid keeps the nucleic acid in place and guarantees that it can make an orderly exit when the time comes for such a process to occur

Outside of the nucleic acid portion is a protein coating made up of 2 parts one a part concerned perhaps with the purely protective purpose of preserving the nucleic acid from the action of enzymes and other agents while the virus ... Finally there

... mportant ... that ... form

of any portion of the entire viru ... as long as a nucleic acid molecule enters ... along with a small quantity of additional material the infection process is ... cerned with infectivity ... cation of the host ... d they form the six features that can be used as keys to understanding the physical action of a viru

A bacterial virus is shown in the center of FIGURE 1 Exactly the same features are present so there is no real need to detail them the diagram is

self explanatory The process of entry of the nucleic acid into the host is perhaps a little more clear-cut and definite in the case of bacterial viruses than it is in the case of animal viruses, but there seems no reason to believe that there is an essentially different entire range of features to separate the two forms of virus

FIGURE 1 (*right*) shows a rodlike plant or insect virus, note that the number of distinctive features is somewhat smaller than for the other two The process of attachment, in the case of the plant virus, is of somewhat doubtful function

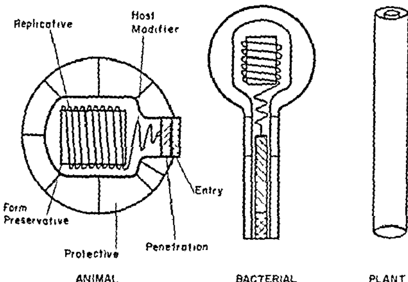


FIGURE 1 Highly schematic drawings of animal bacterial and plant (or insect) virus The functional units are indicated in some detail for the first two For the third (plant or insect) type we know little more than that it contains a nucleic acid in the center of a protein portion

and usually infection must be produced by wounding the cell the question of whether the host modification is necessary is not yet known

Action of Physical Agents

Physical agents act on these various molecular units and alter their function In order to form some theoretical appraisal of the action of physical agents it is therefore necessary to deal with them individually and to consider the effect of each physical agent on these units

Thermal action Thermal action has two features, both of which may be of significance The first is a basic structural alteration of the particle due to the differential expansion of the various parts of the virus under the action of heat Thus, if we consider the form preservative protein as inside the coiled nucleic acid and suggest that the nucleic acid has a molecular weight 10 which would give it a length of 25 000 Å we can see that if this were coiled

on a "spool" of radius 50 Å and had an expansivity of 1.6×10^{-4} per degree centigrade, then a 63° rise in temperature would change the effective length on the spool by 250 Å. This change might very well disrupt the hydrogen bonding and destroy the space relationship that is necessary to keep the structural integrity of the spool and the nucleic acid on it. If this were true the virus would not function as well, and possibly not at all. Evidence for such a process was obtained by Adams,[2] who showed that there is a change in light scattering of T5 phage at the stage where thermal inactivation takes place. This result seemed to correspond to the destruction of the integrity of the nucleic acid within the coat.

Little knowledge is available regarding the thermal expansivities of proteins and nucleic acids. Some early measurements that were auxiliary to sedimentation studies indicated that protein expansion coefficients are high. I have made some measurements on egg albumin showing that a value of 1.6×10^{-4} per degree centigrade is reasonable; this value was used in the above calculation. Ribonucleic acid does not seem to expand nearly as much. The protein expansivity is almost certainly due to some change in the secondary structure as the large value would require 90 per cent of all bonds to be hydrogen bonds; this is certainly not the case. Consequently, some kind of unfolding must occur. Possibly the unfolding cannot occur in nucleoprotein but, at the same time, internal tensions may develop that have the effect of destroying the functioning of the virus.

There is a clear relationship here to another physical method of inactivation, namely, osmotic shock. This condition gives rise to a sudden high internal pressure that disrupts the integrity of the virus, quite possibly without damaging any of the components themselves, but merely separating them from their pools of water.

A second method of operation is the inactivation of the most sensitive part. According to the theory of absolute reaction rates, the inactivation of a protein or nucleic acid follows the relation

$$\frac{dn}{dt} = k_1 n$$

where n is the number of active molecules present, t is time, and k_1 is the reaction constant. In addition,

$$k_1 = \frac{kT}{h} e^{-\Delta S/R} e^{-\Delta H/RT}$$

In this equation there are two terms: the entropy S and enthalpy H of activa-
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best, one should obtain a noninfectious but serologically potent virus on the one hand and a serologically inert but still infectious virus on the other. The proper experimental follow up of this type of lead has not yet been made. An indication that perhaps the entropy and enthalpy values do not vary very

much from component to component is given by some work in my laboratory on T1. Here a very wide range of temperature was employed for the inactivation, and it may be seen that over this wide range the thermal constants remained the same. Were there different values for different units, one would expect that at high temperatures the thermal constants would differ from those found at low temperatures.

One interesting aspect of thermal action could be concerned with the effect of a second agent, such as formaldehyde. If there is a problem of the modification of the protein coat effected by such an agent, then the fact that the thermal expansion of protein is so great could easily mean that openings were formed in the coat as a result of the expansion and that a small molecule such as that of formaldehyde could then more readily enter. There might therefore be a very definite synergistic relationship between formaldehyde and heat. This could possibly account for the differential behavior of the virus at different temperatures.

Ultraviolet light. Ultraviolet light acts predominantly on the nucleic acid. In a secondary way it also acts on protein. In both cases the quantum yield is not very high and, in the case of nucleic acid, the mechanism of which is not yet understood, could be caused by two factors. These two factors are the cross linking of the nucleic acid, which prevents its proper entry into the cell or makes it subject to rupture upon such entry; the other possibility is the breaking of the nucleic acid chain, thus preventing it from producing the specific ribonucleic acid (RNA) for protein when it enters the cell. In the case of protein the effect is a little more clearly understood. Due to the work of Setlow[3] it seems likely that one major effect of ultraviolet light is to cause an S—S bond to become excited, thus rendering it capable of being broken by the action of water or possibly in some other way. Such a rupture can be followed by the denaturation of the protein, which will change its configuration and cause it to cease functioning properly.

In any event the primary result to be expected from ultraviolet action is an effect on a nucleic acid and thus an effect on the infectivity. It should be perfectly possible to inactivate a virus with ultraviolet light, thus yielding a noninfectious but antigenically suitable virus particle. The difficulty lies in the fact that most virus preparations have an appreciable thickness, there is considerable absorption, so that virus particles may well not receive sufficient ultraviolet light to be inactivated by it in the nucleic acid portion before the protein portion is also affected.

There is also evidence from actual studies on ultraviolet inactivation that very often a secondary feature giving rise to an infectious particle can exist that is itself not very sensitive to ultraviolet light. Thus ultraviolet light inactivation does not always follow a logarithmic form over a very wide range, but quite frequently exhibits a tailing off at the end.

Ionizing radiation. The effect of ionizing radiation is that it acts upon both the nucleic acid and the protein. The fact that very frequently the sensitivity of the virus corresponds physically to the sensitivity of the nucleic acid probably reflects the far greater importance of the nucleic acid in the viral function than that of the protein. Thus while a single ionization can inac

either a nucleic acid molecule or a protein molecule, the fact that the entire nucleic acid portion seems to be necessary for the infectious process means that the protein is far less important than the nucleic acid, in spite of the equal in activation. However, there are multiple units for protein, which indeed may not be concerned at all with virus function.

The action of ionizing radiation on nucleic acid is either to break it or to cross link it. FIGURE 2 shows schematically what is believed to occur. Both of these have very important effects, and both are extremely sensitive to ionization. Effectively, a single ionization is likely to cause a break, although there has been some question as to whether, in the case of desoxyribonucleic acid (DNA), there is not a requirement for two ionizations close together to break a two stranded chain. In the case of proteins the inactivation is again likely to be sensitive at the disulfide bond. It seems likely from the theoretical work of Platt and the studies of Gordy *et al*.[2] that the positive charge left behind by ionization can migrate and has, therefore, a chance of settling in the minimum energy region, which is quite often an S—S linkage. Under these

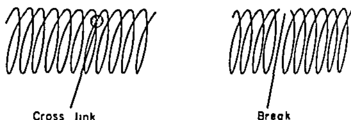


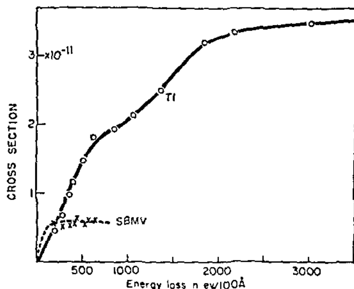
FIGURE 2. Schematic representation of the processes of cross linking and breaking that are supposed to occur in irradiated nucleic acid.

conditions the molecule is very sensitive to subsequent action by water alone by water containing oxygen, or indeed by oxygen itself, the breakage can become confirmed by subsequent chemical action.

It is interesting that there is a strong potentiation of ionizing radiation action by heat, and that it may well be that heat affects the basis of a structural alteration by presenting a somewhat contracted molecule, and that a single ionization can be effective where two were necessary before such contraction.

Light and densely ionizing radiation produce differential effects. This is especially true in virus particles that contain DNA, this is seen most strikingly in the phages. The contrast between the stability of southern bean mosaic virus and T1 phage as regards different ion density radiation is shown in FIGURE 3.

Pressure. The effect of pressure on viruses can be divided into two classes. Where extremely high pressures ranging up to many thousands of atmospheres are employed there is a totally disruptive effect on the virus that is reflected in a loss of activity. Of greater interest is the fact that at lower pressures the relationship to thermal inactivation begins to appear. Thus, if virus is heated while under pressure, the pressure may stabilize the virus. It would seem as though the critical bond that is necessary for the inactivation of the virus may



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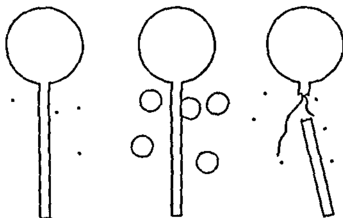


Figure 4 A simplified graphic representation of the manner in which cavitat the disruption of a virus with the consequent exposure of its nucleic acid to the hazards the medium surrounding it

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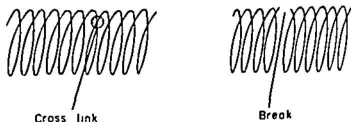


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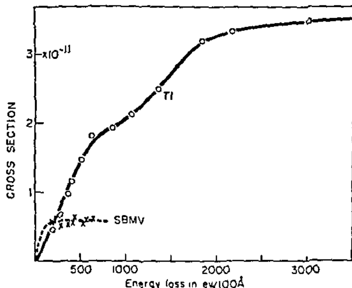


FIGURE 3 The effect of varying linear ion densities on the inactivation of 2 viruses. The [illegible]

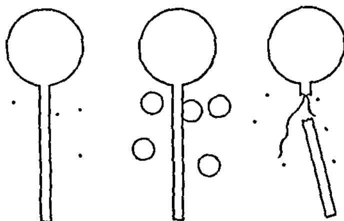


FIGURE 4 A simplified graphic representation of the manner in which cavitation causes the disruption of a virus with the consequent exposure of its nucleic acid to the hazards of the medium surrounding it

very well have a definite increase in stability because of the fact that every part of the molecular structure is held closer while the pressure is exerted resulting in a clear stabilization.

There are marked exceptions to this, and occasionally a virus can actually be less stable under pressure. It appears that work in the field of pressure inactivation, which has had little attention in recent years, should be revived and that, in combination with thermal studies, considerable knowledge about viruses could be obtained very easily. It might very well happen, for example, that a virus heated under pressure would retain the antigenic surface rather more readily than virus that was allowed to expand and break apart thus losing its antigenic surface.

Sonic radiation. The probable effects of sonic radiation are in terms of cavitation, that is, from the rapidly expanding bubble of dissolved gas resulting from extremely rapid alternations of pressure in the vicinity of the source of sound. A schematic diagram is shown in FIGURE 4. Such rapid alternation of pressure can break rodlike structures such as those of tobacco mosaic virus or the tails of bacteriophages. The work done to date indicates that the larger and longer tailed viruses are more sensitive to sonic action. One would feel therefore that sonic inactivation is quite clearly related to structural damage and not so much related to damage to the individual parts, as may be seen for one facet of thermal action and, definitely, for the action of radiation.

Osmotic effects. The high osmotic pressure produced inside a virus particle that has previously been exposed to a small solute of high concentration and then placed in distilled water almost certainly produces rapid changes in pressure, thus producing active disruption of the virus. Such preparations often release nucleic acid quite visibly, and inactivation is related to this phenomenon. It is quite likely that much of the nucleic acid is still intact, but the auxiliary problem of the entry into the cell now must be solved by nucleic acid which exists precariously in the presence of nucleases and other disruptive agents. The result is effectively an inactivation.

Conclusion

Physical methods of inactivation have not received the attention they deserve. The methods themselves are quite diverse and, as they become better understood, they should be most informative about the nature of the virus and its action. The fundamental studies on processes of inactivation are, nevertheless, progressing, although not nearly as far as we might wish. As these fundamental studies go forward, the use of physical agents both in applied virology for sterilization purposes and for the production of vaccines, should increase. At the same time it is to be hoped that pure virology will benefit from the interpretations that will be possible when inactivation is followed as an understood process.

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EFFECTS OF NONIONIZING RADIATION ON PLANT VIRUSES

A. Kleczkowski

Rothamsted Experimental Station, Harpenden, Hertfordshire, England

Limitations of space do not permit a full review of the subject indicated by the title of this paper, therefore, only a few selected problems are discussed. These are concerned chiefly with the kinetics of inactivation of plant viruses by ultraviolet (UV) radiation, photoreactivation, and protection of nucleic acid by union with protein.

Kinetics

It seems that all viruses are inactivated by UV approximately according to the first-order kinetics. This means that straight lines are obtained if logs of remaining proportions of the original infectivity are plotted against doses of irradiation. This is usually interpreted as evidence that virus particles are inactivated by single quanta of the radiation that happen to hit some part of the virus that is essential for activity, the hypothesis of active center. Although each particle usually absorbs many quanta before it is inactivated, it is nevertheless assumed to be inactivated by one of the quanta that happens to hit the active center. This single hit hypothesis is treated by many authors as if it were firmly established. However, as this hypothesis may often influence conclusions drawn from results of various irradiation experiments and consequently, may affect further experimental designs, it seems desirable that attention should be drawn to the fact that this hyp[illegible] l, consequently, other possi[illegible]

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The argument upon which the single hit hypothesis rests is statistical and is based on the Poisson series. If the mean number of inactivating hits per particle is, for example, m, the proportion of particles that would have received 0, 1, 2, 3, and so on of such hits would be equal, respectively, to the successive terms of the Poisson series, which are,

$$e^{-m}, \quad m e^{-m}, \quad \frac{m^2}{2!} e^{-m}, \quad \frac{m^3}{3!} e^{-m}$$

and so on. Now, if x is the dose of radiation energy given to a virus solution m would be proportional to x. Let $m = kx$ where k is a constant. Thus if each virus particle has only one active center and if one hit on the center is sufficient to inactivate the particle, the particles that would remain active would be only those that received no hit on the center and the proportion of such particles will be $p = e^{-m}$. Plotting the log of p against m gives a straight line, therefore, the inactivation would obviously follow the first-order kinetics.

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As discussions on this subject usually lead to misunderstandings I shall try to show here as clearly as possible why with the evidence at present available the single hit hypothesis cannot be considered as definitely established To do this I must cover some ground that may be obvious and elementary but this is unavoidable

The argument upon which the single hit hypothesis rests is statistical and is based on the Poisson series If the mean number of inactivating hits per particle is for example m the proportion of particles that would have received 0 1 2 3 and so on of such hits would be equal respectively to the successive terms of the Poisson series which are

$$e^{-m} \qquad m e^{-m} \qquad \frac{m^2}{2!} e^{-m} \qquad \frac{m^3}{3!} e^{-m}$$

and so on Now if v is the dose of radiation energy given to a virus solution m would be proportional to v Let $m = kv$ where k is a constant Thus if each virus particle has only one active center and if one hit on the center is sufficient to inactivate the particle the particles that would remain active would be only those that received no hit on the center and the proportion of such particles will be $p = e^{-m}$ Plotting the log of p against m gives a straight line therefore the inactivation would obviously follow the first-order kinetics

If we assume that inactivation of a particle is a result of hitting the same

target at least n times, or each of n separate targets at least once or at least r times, the conclusion would be that the course of inactivation would not follow the first-order kinetics for the proportions of particles still remaining active would then be, respectively

$$p = e^{-m}\left(1 + m + \frac{m^2}{2!} + \frac{m^3}{3!} + \quad \frac{m^{n-1}}{(n-1)!}\right)$$

or

$$p = 1 - (1 - e^{-m})^n,$$

or

$$p = 1 - \left\{1 - e^{-m}\left[1 + m + \frac{m^2}{2!} + \frac{m^3}{3!} + \quad \frac{m^{r-1}}{(r-1)!}\right]\right\}^n$$

Plotting logarithms of p given by any of these 3 equations against m will not give a straight line

The conclusion of all these considerations is that if inactivation by ultraviolet radiation were a result of a certain definite number of hits on a certain definite number of active centers (targets) in each virus particle the fact of inactivation following the first-order kinetics could be reconciled only if each of these numbers were 1, and this would agree with the single hit hypothesis However is it necessary to assume that any part of a virus particle must absorb any definite number of quanta for inactivation to occur?

All that can be concluded definitely from the fact that the first-order kinetics is followed is that when a dose r of the radiation is given, the probability of survival for each virus particle is $p = e^{-kr}$, where k is a constant Irradiation may result in a condition that could be called the state of disequilibrium which could be imagined as the presence of excitations in various parts of a virus particle with a possibility that a change resulting in inactivation may occur The intensity of the condition would depend on the rate of absorption of radiation energy Inactivation may occur at any particular moment while the condition lasts, and no definite number of quanta need be absorbed by any particular part of a virus particle for inactivation to occur The disequilibrium hypothesis seems plausible because of the variety of possibilities following the absorption of a quantum by a virus particle The quantum energy may be transferred from one site to another, it may cause either a temporary or a permanent alteration in a detail of structure that may or may not be essential for infe [illegible] and be dispersed

The [illegible] by the fact of inactivation followir [illegible] to be no other evidence in favor of the hypothesis whereas there are a few facts that appear to contraindicate it First the inactivation of some viruses deviates appreciably from the first order kinetics For example, the *Escherichia coli* phage T2 shows a deviation at the very beginning of inactivation[1] This observation is rather difficult to reconcile with the single hit hypothesis a constant target should be there from the beginning By the disequilibrium hypothesis on the other hand the initial deviation would simply mean that the condition of disequilib

num takes some time to develop to the degree of intensity that corresponds to the intensity of the radiation

A second fact that is contrary to the single hit hypothesis is the extreme smallness of quantum yields usually obtained with plant viruses. For example, a particle of tobacco mosaic virus (TMV) absorbs, on the average, about 25,000 quanta of the radiation of 2537 Å before it is inactivated[23]. The single hit hypothesis implies that absorption of radiation energy by any part of the virus particle other than the hypothetical active center of the size of, for example, one pyrimidine ring does not affect the activity of the particle. One must assume, therefore, that infectivity of a virus particle depends entirely upon the integrity of such a small part of it as a single pyrimidine ring that would then have to be the sole carrier of all of the so called genetic information, and this seems unlikely. The disequilibrium hypothesis, on the other hand, is supported by the fact of the smallness of quantum yields.

None of the above considerations can be taken as sufficient evidence for the rejection of the single hit hypothesis; the hypothesis may be true, but it cannot be considered as definitely established.

Photoreactivation

Whenever the phenomenon of photoreactivation occurs, it allows us to distinguish between two different kinds of UV irradiation injury: the kind that is reversible by photoreactivation and the kind that is not. Not all plant viruses show the phenomenon of photoreactivation. With those that do, it occurs when plants inoculated with UV irradiated virus preparations are exposed to visible light for a few hours after inoculation. To prevent photoreactivation, the inoculated plants must be kept in darkness for a few hours after inoculation.

Whenever the course of inactivation of a plant virus by UV was investigated it appeared to approximate the first-order kinetics irrespective of whether photoreactivation did or did not occur, although the rate of inactivation appeared reduced by photoreactivation[4]. Photoreactivation is equivalent to a reduction of the dose of irradiation by a constant factor characteristic for a given virus; this is the so called dose reduction principle formulated by Kelner for cellular organisms[5]. Thus, after applying the dose v of UV radiation, the proportion p of remaining active virus is, for example, $p = e^{-k_{dark} v}$ without photoreactivation and, for example, $p = e^{-k_{light} v}$ with photoreactivation. The ratio k_{dark}/k_{light} gives a measure of photoreactivation, which differs from one virus to another. TABLE 1 shows the values of the ratio obtained with several plant viruses. The ratio of 1 for different strains of tobacco mosaic virus shows that with this virus there was no photoreactivation. The highest ratio of about 3 was obtained with potato virus X. Although TMV showed no photoreactivation, the infective nucleic acid isolated from TMV did so, the k_{dark}/k_{light} factor being about 2.

That photoreactivation actually is a reversal of a damage inflicted upon a virus particle by UV, and not just an avoidance of the result of the damage was shown by Lennox *et al* for the photoreactivation of a bacteriophage. No comparable evidence has been obtained with a plant virus, but the sion is probably applicable to plant viruses for the phenomenon of pho

ation seems to be of a general nature We assume therefore that there are two different kinds of damage, one being reversible by photoreactivation the other irreversible The question now arises Are the two kinds of damage quite independent of each other, or is the irreversible damage a further stage of the reversible kind of injury?

We assume that each process is a first-order reaction If we assumed that

order kinetics On the other hand if we assume that the two kind of changes occur quite independently of each other, the expected proportions of particles remaining active with or without photoreactivation would agree with the first

TABLE 1

RELATIVE AMOUNTS OF PHOTOREACTIVATION WITH DIFFERENT VIRUSES

Virus	k_{dark} / k_{light}
(1) Tobacco mosaic (TMV)	
(a) Stock strain	1 0
(b) Datura strain	1 0
(c) U1	1 0
(d) U2	1 0
(e) Tomato aucuba	1 0
(2) Tomato bushy stunt (BSV)	1 2
[illegible]	1 2
[illegible]	1 5
[illegible]	1 9
[illegible]	2 0
(7) Potato X	3 1
(8) Nucleic acid isolated from the stock strain of tobacco mosaic virus	2 0

order kinetics It seems therefore, that the two kinds of changes are quite independent of each other

Let the proportion of particles that have not undergone the first (reversible) change be $p = e^{-k_1 v}$ Thus the proportion that have undergone the change would be $q = 1 - e^{-k_1 v}$ When the dose of the radiation is increased by an infinitesimal increment dv the infinitesimal increment of the proportion of changed particles would be $dq = k_1 e^{-k_1 v} dv$ Therefore if the total dose of the radiation is going to be w the proportion of the increment that will not undergo the second (irreversible) change would be $e^{-k_2(w-v)}$ Consequently the amount of the increment that will not undergo the second change would be

$$e^{-k_2(w-v)} dq = k_1 e^{-k_2 w} e^{(k_2 - k_1)v} dv$$

Thus, the total proportion of virus that would undergo the first change but not the second one would be

$$p_r = k_1 e^{-k_2 w} \int_0^w e^{(k_2 - k_1)v} dv$$

If the second change is a further stage of the first change, it cannot occur in particles that did not undergo the first change, and the proportion of these is $p_s = e^{-k_1 w}$ Thus, the total proportion of virus that did not undergo the second change would be

$$p = p_s + p_r = e^{-k_1 w} + k_1 e^{-k_2 w} \int_0^w e^{(k_2 - k_1)t} \, dt$$

If $k_1 = k_2 = k$, the solution is $p = e^{-kw} + kw\, e^{-kw}$ If $k_1 \neq k_2$ the solution becomes

$$p = \frac{1}{k_2 - k_1} (k_2 e^{-k_1 w} - k_1 e^{-k_2 w})$$

Either of these solutions would give the proportion of virus remaining infective when the reversible change has been reversed that is when photoreactivation has taken place Neither of these solutions agrees with experimental results, for neither can be transformed into the form $p = e^{-k_{dark} w}$ which does agree

If, on the other hand the two changes are assumed to be independent of each other, the proportion of virus that has not undergone the irreversible change would be $p_2 = e^{-k_2 w}$ This would be the proportion remaining infective when photoreactivation has occurred therefore k_2 would equal k_{light} The proportion of virus that has not undergone the reversible change would be $p_1 = e^{-k_1 w}$ Thus, the proportion that has not undergone any change would be $p = p_1 p_2 = e^{-(k_1 + k_2)w}$ This would be the proportion remaining infective when photoreactivation has not occurred Thus $(k_1 + k_2)$ would equal k_{dark} The assumption is, therefore, compatible with experimental results

Photoreactivation of an UV irradiated plant virus can take place during a limited period of time that starts some time after the host plant has been inoculated This phenomenon has been investigated with potato virus X, using tobacco as the host plant [6] At the temperature of the greenhouse (about 20° C) it makes little difference whether the inoculated plants are exposed to light or kept in darkness during the first half hour after inoculation After that period has passed most photoreactivable virus becomes photoreactivated if the plants are exposed to ordinary daylight for about 15 min If, however, the plant is not exposed to light during the next hour, most virus ceases to be photoreactivable and becomes permanently inactive Thus, there are two changes in the condition of a virus particle that is reversibly inactivated by UV The first change occurs on the average about one half hour after the inoculation of the host plant and it makes the particle ready for photoreactivation If photoreactivation does not take place during the next hour, the second change occurs which makes the particle permanently inactive The particle may become decomposed by the host It must be added, however, that no comparable study has been made with any plant virus other than potato virus X and so we do not know how general this sequence of phenomena may be

Inhibition by UV Inactivated Viruses

With some bacteriophages and some animal viruses the damage caused by UV can be repaired to some extent by the process known as multiplic

reactivation when virus particles that are inactive singly can apparently cause infection when several of them enter the same host cell.[7,8] This was deduced from the fact that, when the concentration of UV irradiated virus increased, the residual infectivity increased more than proportionately.

UV irradiated preparations of TMV were tested for multiplicity reactivation but none was found.[4] The infectivity of irradiated virus preparation, instead of increasing more than proportionately with the increased concentration of the inoculum, actually increased less than proportionately because of the inhibition by inactive virus of infectivity of remaining active virus.

The phenomenon of inhibition of infectivity of active virus by UV inactivated virus is of importance not only because of its intrinsic interest but also

TABLE 2
INTERFERENCE OF INACTIVE WITH ACTIVE VIRUSES

Exp. No.	Contents of inocula (mg./l.)		Total numbers of lesions on 12 half leaves	Host plants
	Untreated virus	Inactivated virus		
1	0.2 TMV	5000 TMV	30	*Nicotiana glutinosa*
	0.2 TMV	5000 BSV	104	
	0.2 TMV	—	119	
2	0.5 BSV	5000 TMV	38	
	0.5 BSV	5000 BSV	48	
	0.5 BSV	—	48	
3	0.2 TMV	4000 TMV	52	
	0.2 TMV	4000 RTNV	139	
	0.2 TMV	—	196	
4	0.2 RTNV	4000 TMV	107	French bean
	0.2 RTNV	4000 RTNV	44	
	0.2 RTNV	—	193	

because it may affect results of infectivity tests with irradiated virus preparations and so lead to false conclusions about the kinetics of inactivation.

Three different plant viruses were tested in this respect: TMV, tomato
TNV).[4]
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centration of the inactive virus in the inoculum was higher than ... per cent. This concentration is rarely exceeded when the kinetics of virus inactivation by UV is investigated but, whenever it is exceeded, the inhibitory effect of the inactive virus must be taken into consideration.

When the inhibitory effect is shown, it varies in detail from one virus to another. TABLE 2 shows that, when TMV is compared with RTNV, the inhibitory effect shows a high degree of specificity. Active TMV was inhibited

strongly by inactivated TMV and only very slightly by inactivated RTVV and vice versa Inactivated BSV, on the other hand showed only a very slight inhibitory effect on either active BSV or TMV and no specificity could be noticed

Specificity in the inhibitory effect can be interpreted as an indication that the irradiated virus particles may still be able to engage specific receptor sites in the susceptible host cell in the same manner that the active virus does when it becomes established to multiply, although the ability of the irradiated virus to do so has been lost The unspecific inhibitory effect that may be shown either in addition to or without the specific effect is probably of the same kind as the inhibitory effect of almost any protein that may be added to the inoculum Plant viruses are very susceptible to the unspecific inhibitory effects of many kinds of proteins This may result from some kind of mechanical interference with the movement of virus particles within the host plant or from upsetting the metabolism of the host cell

Protection of Nucleic Acid by Protein

As nucleic acid seems to be the carrier of infectivity the inactivation of infectivity of a virus by UV presumably is a result of an alteration in the structure of the nucleic acid by UV The protein may play a part by affecting the rate of inactivation however because the union with the protein within a virus particle can give to the nucleic acid a considerable degree of protection against the radiation The protection comes only to a small extent from a shielding effect of the protein but chiefly from making the nucleic acid itself more resistant to inactivation According to the results of McLaren and Takahashi[10] about 5 times more radiation energy per unit weight must be absorbed by the nucleic acid within the intact particle of TMV than by the isolated nucleic acid of the virus to reduce infectivity to the same proportion of the original Siegel *et al*[11] gave evidence that a decrease in susceptibility of virus nucleic acid to UV depends on the way the nucleic acid is combined with the protein They found that the infective nucleic acids isolated from 2 different strains of TMV (U1 and U2) did not differ from each other in susceptibility to UV radiation although the 2 original virus strains differed considerably from each other in this respect These authors concluded that the difference in the sensitivity of the two [virus] strains to inactivation with ultraviolet irradiation resides in the nature of the bonding between nucleic acid and the protein moieties of the virus [11]

The results of all these comparisons between the rates of inactivation of TMV and of its isolated nucleic acid can be interpreted in 2 different ways (1) inactivation of the nucleic acid by UV may be a result of any one of a number of different kinds of injury the union with the protein protecting the nucleic acid entirely against some kinds of injury and (2) there is only one kind of injury the union with the protein making it require more energy Evidence that there are at least two different kinds of injury comes from the phenomenon of photoreactivation but the phenomenon is not shown by TMV[4,6] Evidence has been found[2] that this is the case because the nucleic acid within the intact virus particle is protected from the kind of injury that

is reversible by photoreactivation This is concluded from the fact that the isolated infective nucleic acid of the virus shows the phenomenon of photoreactivation but only if irradiated with UV after isolation from the virus and not if irradiated as a part of the virus and then isolated

The amount of energy that must be absorbed by the isolated nucleic acid to reduce its infectivity by one half is about 0 4 joules per milligram with photoreactivation and only about one half of this without photoreactivation Thus, one half of the absorbed radiation energy causes the kind of injury that is reversible by photoreactivation and does not seem to occur at all when the nucleic acid is combined with the protein in the original virus particle According to the results obtained by McLaren and Takahashi,[10] about 2 0 joules of the radiation energy must be absorbed per milligram of the nucleic acid within the original virus to halve its infectivity, which is about 10 times the amount needed to halve the infectivity of the isolated nucleic acid when photoreactivation does not operate This can be interpreted to mean that about 90 per cent of the energy absorbed by the nucleic acid inside the original virus is prevented from causing injury because of the protecting effect of the protein and this includes the 50 per cent of the energy that would have caused the kind of injury that is reversible by photoreactivation

We do not know how protein can protect nucleic acid from injury by UV The protection may be based on increasing the rigidity of the structure of nucleic acid by bonding with protein or on preventing photolysis by excluding access of water to parts of nucleic acid ... f TMV does

to UV shown by the original virus

Effect of UV on Host Plants

UV irradiation of host plants decreases their susceptibility to infection with viruses and this effect can be reversed to a varying extent by photoreactivation if the amount of UV irradiation has been kept within limits [9,13,14] However this subject has been but little investigated The effect of UV on the host plant may interfere when it is intended to study inactivation of a virus by UV within the host

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EFFECTS OF NONIONIZING RADIATIONS ON ANIMAL VIRUSES

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This paper considers certain aspects of recent research pertaining to the practical application of ultraviolet irradiation in the inactivation of viruses for the preparation of vaccines We shall be concerned chiefly with tissue culture fluids, tissue extracts virus concentrates, and the equipment and apparatus developed for effective irradiation under controlled conditions

The term inactivation of viruses in its broadest sense means the loss of all biological activity, but in the sense used here it refers to the destruction of the capacity of the viruses to infect and reproduce, that is, to cause disease Other biological properties especially the capacity of acting as an antigen to produce antibodies must be retained This property is, in many cases the only means of testing for biological activity after the infectious capacity is removed

In recent years research on the application of ultraviolet in the production of virus vaccines[1,2] has shown that the objective originally desired such as the complete destruction of infectivity by means of ultraviolet irradiation alone would not be practicable with all viruses Rabies virus is an example of an extremely sensitive agent, and complete inactivation by means of ultraviolet with the retention of antigenic potential is feasible[3,4] (also Kay, Oppenheimer and Taylor *unpublished data*) *Extensive studies with irradiated poliovirus* vaccines[5-13] and the influenza viruses[14,15] (McLean and Taylor unpublished data) have shown however, that irradiation used in combination with other inactivating agents[2,8,10] is much more effective It provides not only more highly antigenic vaccines but also more consistent and reliable inactivation Furthermore the stability of such products is superior to those produced by either chemical inactivation or irradiation alone Some discussion of the

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vealed[1,8,16] *that there were at least two possibly more,* distinct stages in formaldehyde inactivation of this virus It appeared quite probable that a combination of chemical and thermal reactions was involved This delay in the initiation of an infection phenomenon has been confirmed by Gard and his co workers and is discussed elsewhere in this monograph

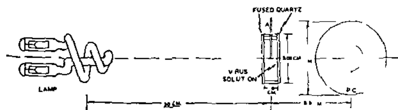
Effects of ultraviolet or nonionizing radiations on viruses have been reviewed by Pollard[17] in 1953, and more recently by Kleczkowski[18] In addition many newer theoretical and integrated concepts are discussed elsewhere in these ... waves

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is considered to be due to the absorption of the ultraviolet photons within nucleic acids or nucleoproteins The proteins with aromatic side chains and the polypeptide portions of virus particles also absorb but less efficiently In general many thousand quanta of ultraviolet energy are absorbed before a virus particle is inactivated [17, 9]

Experimentally a pure suspension of viruses in a nonabsorbing medium may be treated with a given amount of incident ultraviolet energy that is determined by physical measurement From this the total amount of energy absorbed by the suspension in a given time can be calculated The total energy absorbed then may be divided by the total number of organisms or by the total volume of protoplasm involved to obtain the net energy absorbed per organism or per unit volume If this is correlated with biological tests a definite ina tiv ig dose of ultraviolet can be formulated Practicably such condition are ex e l ingly difficult if not impossible to achieve Many variable factor a it l



with biological suspensions preclude the exact determination of the effective proportions of any given quantity of incident ultraviolet energy applied

Conditions must be established for each individual virus preparation so that a safe minimum of ultraviolet energy can effect consistently the desired biological action A suitable biological safety test is the final index of inactivation an antigenic potency test determines the usefulness of the product A certain amount of energy intense enough to affect the viruses must reach every portion of the suspension and the net photon density throughout the fluid should vary within only a very small range The application of ultra v le e

or open dishes [22] and the depth of the fluid to be irradiated varied over wide limits (FIGURE 1) Even with the expedient of stirring the suspensions data

titer) is plotted against the time of exposure and also against the dosage[1] of ultraviolet (infectivities were measured by plaque, roller tube, or plastic plate techniques) None of the curves follows a constant exponential course, the initial increment produces an average log loss in titer of 2 25, the successive



FIGURE 3 The completely assembled Centrifilmer unit is approximately 36 inches in over all length and weighs 120 lb Reproduced by permission from American Journal of Public Health and The Nation s Health[4]

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inactivation was much more rapid and appeared to be exponentia probably due to the reduction in film thickness In addition the inactivation rate curves could be made apparently linear by dilution of the tissue-culture virus fluids with saline (1 4 or 1 8) Neither the reduction of the w rate nor the

dilution of the medium was of practical importance, both procedures resulted in reduced antigenic potential of the preparations.[5]

Inactivation rate and ultraviolet absorption spectrum studies were made also on virus concentrated (×20) by cold alcohol precipitation (FIGURE 6). Con-

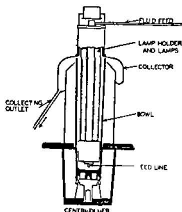


FIGURE 4. Diagrammatic vertical section of centritugal ıılnı C trıblmer for the exposure of fluids to ultraviolet irradiation in the form of thin fl w nᵘ h ıms. Reproduced by permission from *The Journal of Immunology*.[6]

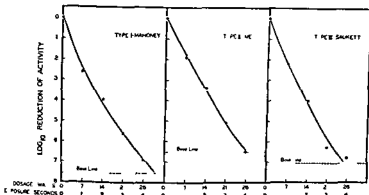


FIGURE 5. Inactivation rate curves of 3 types of poliomyelitis virus using successive 7 watt increments of incident ultraviolet energy. Reproduced by permission from *The Journal of Immunology*.[6]

centrated poliovirus in buffered saline solution, ultraviolet treated by using 7 watt increments, revealed the same nonexponential relation seen with the tissue-culture fluid virus. Here, however, some of the nonlinearity probably was caused by the absorption of ultraviolet energy by the virus itself.[6,21] Comparison of the ultraviolet absorption spectra of irradiated and unirradiated virus (FIGURE 7) showed that irradiation produces a shift of the characteristic

absorption maximum at 260 $\mu\mu$ toward the shorter wave lengths. At the same time there is an increase in the absorption from wave length region of 330 to 280 $\mu\mu$. This latter change can be associated with the protein moiety of the virus. Thus, in these relatively concentrated and partially purified poliovirus suspensions both the nucleic acid and protein portions of the virus are affected. Similar effects were observed in earlier virus inactivation rate tests with equine encephalomyelitis (EE) virus[21] (FIGURE 8). The rate of loss

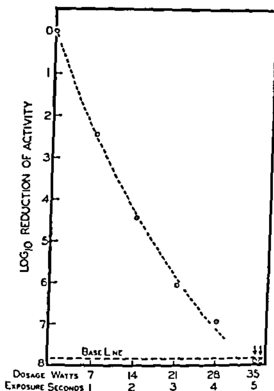


FIGURE 6. Inactivation rate curve of 20X concentrated Type I (Mahoney strain) virus in buffered saline solution. Medium absorption effects are negligible; the nonexponential relation is due to the ultraviolet absorption of the virus. Reproduced by permission from *The Journal of Immunology*.

of infectivity of this virus for dilute solutions (0.01 mg/cc) was linear and this would indicate a primary photochemical reaction. With virus protein concentrations of 0.02 mg/cc, however, the rate of inactivation decreased with time. Paralleling this decrease in the rate of inactivation there was a progressive increase in ultraviolet absorption with time of irradiation. This decrease in the rate of inactivation of the more concentrated solutions has been considered as due, at least in part, to the increase in ultraviolet absorption.

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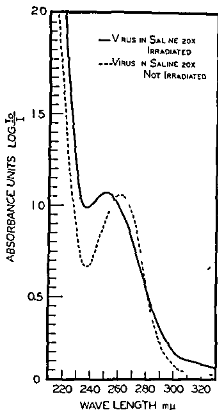


FIGURE 7 Ultraviolet absorption spectra of irradiated and nonirradiated 20X concentrated Type I (Mahoney strain) virus The maximum and minimum absorptions are both altered following irradiation Reproduced by permission from *The Journal of Immunology*

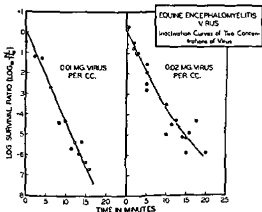


FIGURE 8 Rate of inactivation of equine encephalomyelitis virus protein in Ringer solution irradiated continuously by ultraviolet rays 2537 Å 252 ergs/sq cm /sec Reproduced by permission from *The Journal of Infectious Diseases* [31]

as the irradiation progressed. This could be interpreted to mean direct protein denaturation, aggregation, or even attendant heat denaturation (long exposures). The interference of inactive virus with residual active virus in the titrations used to measure the rate of loss is still another consideration, and an extremely important one.

As suggested by Fogh,[22] the departure from linearity observed with polio-

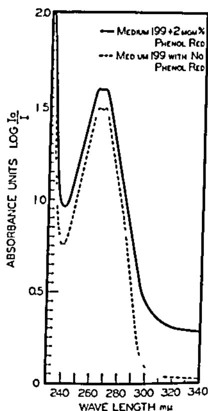


FIGURE 9. [illegible] with and without phenol red [illegible]

virus might be caused by the presence of virus particles that are especially resistant to the irradiation. However, despite many attempts, no such resistant strains have been isolated. The relative over all absorption of ultraviolet by the medium is known to be operative and to affect the inactivation rate. FIGURE 9 shows that the absorption spectrum of medium 199, containing nucleic acid and other protein constituents, was far greater than that of the virus itself. The absorption maximum occurs in the 260 $\mu\mu$ region. This observation serves to emphasize the advantages of the thin film principle coupled with high ultraviolet energy emission for the effective irradiation of

infectious materials in biological fluids especially, it indicates the need for a careful consideration and evaluation of the absorption characteristics of the medium constituents

No attempt has been made to treat mathematically any of the inactivation rate data obtained in these poliovirus experiments In order to calculate the absolute quantity of energy involved in the virus inactivation itself there must be some manner of quantitating the relative amounts of ultraviolet energy absorbed by the virus and by the culture medium Thi ha not been possible as yet furthermore, the absolute exposure time is dependent upon a number of variable factors viscosity, temperature surface tension and frictional resistance to flow Detailed studies of film thickness measurement and the associated factors are in progress and will be reported

Discussion

Critical examination of virus inactivation rate curves such as those shown here will reveal that, within the limits of the error of the virus titrations uniformity of effect is obtainable from one large lot of virus to another With the

exposure were required with the attendant over exposure of much of the virus This thin film principle of irradiation combined with chemical an l heat inactivation has been used for a number of years for the preparation of v ru vaccine that are relatively more highly antigenic and stable than those nact vate l with chemicals alone Some additional basis for this combined inact v t o procedure approach is as follows

It has been stated[7] that the various studies on the inactivation of pol viruses by means of ultraviolet irradiation for vaccine production[7 8 1 3 3 r] have contributed nothing new to our understanding of the kinetics of inactivation by ultraviolet irradiation This contention is only partially true The point of view and the method of approach must be considered If specific detailed information relative to the specific reactions of a given virus is sought the approach must be the careful one of the theoretical chemist The literature contains, however, much pertinent information concerning the effects of

being isolated in increasing numbers from living organisms Also the important factors of coenzymes that contain bases remain to be cons dered The act on [illegible] of ltraviolet rrad ation

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complicating effects and should be ruled out. The irradiation products in some cases have been isolated and analyzed,[25, 26] irradiation in alcohol water mixtures or aldehydes results in loss of characteristic absorption patterns to give nonreversible products.

Specifically, the absorption spectra of nucleotides containing uracil and cytosine, unsubstituted in the 5 and 6 positions, show a loss of 260 μμ absorption. A single hit is suggested, and the change observed is reversible upon treatment with acid or alkali or heat, if the irradiation is not carried too far. The cytosine photoreaction product is more unstable than the uracil product, it has been suggested that either the ring is opened or the 5—6 double bond is saturated. If the reaction were saturation of the double bond, it is probably due to the addition of water from the solvent. Analyses indicate that this is the case,[25, 26] a more drastic effect upon the absorption would be expected if the bond were opened. The infectivity of the free nucleic acid of tobacco mosaic virus (TMV) is 6 times as sensitive to ultraviolet as is the intact virus. If the virus is dried before irradiation, however, its sensitivity to ultraviolet becomes the same as that of the free nucleic acid. Sinsheimer (unpublished data) suggests that in the intact virus the RNA is held in such a position that the pyrimidine nucleotides, after absorption of a photon, can react with solvent in a reversible and possibly noninactivating manner. In the free TMV nucleic acid or in the dried virus, however, this reaction is not possible, and the irradiated nucleotide reacts with a neighboring nucleotide or protein component to produce an irreversible reaction.

No such detailed studies are available as yet for animal viruses, such as polio. However, the concept that the protein shell of the intact virus exerts a protective or tempering action from the irradiation is an attractive one. This concept becomes even more tenable when it is considered that ultraviolet and formaldehyde (or heat) inactivation used in conjunction produce an inactivating effect that is synergistic and irreversible.[2] In all probability the mechanism of virus inactivation by ultraviolet involves the alteration of some molecular organization within the virus particle that is quite different from the mechanics of formaldehyde inactivation[6, 16] (Schaffer, elsewhere in this monograph). The latter may be considered a progressive chemical denaturation by increased bonding occurring predominantly in the protein portion of the virus; the former is a physical reaction, a rupture or rearrangement, or both, but most probably it involves the saturation of double bonds[25, 26] in the nucleic acid portions of the virus. The work of Shugar and Wierzchowski[27] also supports the same trend of thinking and suggests that such studies are important in relation to the biological activities of viruses.

Such information as that discussed above has been reasoned deductively into a working hypothesis from which it has been possible to evolve a consistent and reliable inactivation process for poliovirus vaccines. There are still many fundamental and theoretical details to be worked out, but it would appear justifiable to utilize all available information in the best possible practical manner. This is becoming even more pertinent with the increasing volume of information relating viral infection to the nucleic acids.

In conclusion, it should be pointed out that ultraviolet irradiation is becom

ing increasingly useful in the study of neoplastic tissues and their subcellular constituents The unraveling of the biological interrelations (enzymatic, immunological, physical, and chemical, perhaps even virology) of such cells and their components, both in the naturally occurring state and in tissue culture will require a concerted attack on this very complex problem at the most fundamental level the cell itself Ultraviolet radiation may be used as a stress technique to increase or retard the growth of malignant cells



FIGURE 10 Electron micrograph of an electrophoretically purified hemolysin fraction of S 180 mouse tumor The particles are somewhat variable in size around a mean of 56 $\mu\mu$ ± 12 $\mu\mu$

It is being used to exclude extraneous viruses and to reduce nonspecific toxicities of sera used in media (LoGrippo, elsewhere in this monograph) The early work on the action spectra of several viruses[38] revealed that Rous sarcoma virus was remarkably sensitive to small quanta of ultraviolet energy It has been shown that specific complement fixing antigens can be isolated from such tissues[39] by Genetron treatment and fractionation Viruslike particles extracted from S 180 mouse tumors (FIGURE 10) have been associated with a hemolysin This hemolysin produces a cytopathogenetic effect on mouse embryo tissue culture cells It will al o produce a rapid metaplastic growth in the chick embryo membrane and in the hamster cheek pouch (Truffelli Smith and Taylor, unpublished data) The tumor producing activity of such

grown malignant cells, increases in quantity with repeated passages[40] This fraction is related intimately in some way with the larger virallike bodies that are being demonstrated with great regularity in thin sections (and extracts) from malignant tissues and cells[41-42] Since these fractions contain greater or lesser proportions of nucleic acids it would seem obvious that they should be studied by means of ultraviolet irradiation, spectrophotometry, and electron micro copy Such an approach should yield fundamental information useful in studies of the metabolism of neoplastic cells and in the development of chemotherapeutic testing methods

Acknowledgments

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INACTIVATION OF BACTERIAL VIRUSES BY PHYSICAL METHODS

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Physical methods for the inactivation of bacterial viruses are numerous and varied Heating, shaking, drying, sonication, radioactive decay, and exposure to ionizing or nonionizing radiations constitute only a partial list of the treatments that can cause loss of bacteriophage viability Some methods by which inactivation can be brought about are of no particular importance beyond the fact that they should be avoided in routine experimentation, but others, such as irradiation, are of considerable biological significance No attempt is made here to deal in turn with the various methods The discussion is confined instead, to studies only of phage inactivation by ultraviolet (UV) light and decay of radioactive phosphorus The mechanisms of action of these agents

of inactivation of a phage particle or phage infected bacterium will be the loss of the ability to generate a plaque on a plate culture of indicator bacteria

Inactivation of Bacteriophage Particles

First let us consider the instability of phage particles that contain radiophosphorus (P^{32}) of high specific activity With each of the phage strains examined to date, the kinetics of inactivation of a stock of radioactive particles is described by the equation $\log s = -kf$, where s is the fraction of the particles still viable k is a constant that is peculiar to the phage population, and f is the fraction of P^{32} atoms decayed at the time of assay Thus, the plot of the logarithm of the surviving fraction of particles versus the dose of the inactivating agent (fraction of P^{32} atoms decayed) is a straight line, which shows that the particles in the population are equally sensitive to inactivation by radioactive decay and that each can be inactivated by a single radioactive disintegration The constant k includes three parameters first, Ao, the specific radioactivity of the medium in which the phage particles were grown, second, N, the number of phosphorus atoms per phage particle, and third, α, the probability that a disintegration will inactivate the particle in which it occurs (the efficiency of killing) The parameter of greatest consequence here is α for in the larger phages, including the virulent T-type coliphages, the temperate coliphage λ the *Salmonella* phage P22, and the phage BM that is active on *Bacillus megatherium*, the efficiency of killing at ordinary temperatures is approximately 0 1[1-5] In other words, for each of these different phage strains whose particles contain about 10^{-14} mg of phosphorus, only 1 disintegration in 10 is lethal However, in 2 very small phages, S13 and φX 174, which are active on *Escherichia coli* and contain approximately 100 times less phosphorus than the phages already mentioned, the efficiency of killing is about 10 times greater and very close to 1 0[6,7]

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An explanation of the fact that many different phage strains are inactivated by P^{32} decay with about the same low efficiency is found in a model of lethal action that was proposed by Stent a few years ago[4,5] The original work of Hershey *et al*[1] and other studies[2,3] had shown that a short range effect of the transmutation $P^{32} \rightarrow S^{32}$ rather than the radiation emitted on radio[illegible]tive decay is the main cause of phage death It was also apparent that lethal di[illegible]integra[illegible]tions must occur within the deoxyribonucleic acid (DNA) moiety of mature phages since most, if not all, of the phosphorus of the infectiou[illegible] particle [illegible] accounted for in its complement of nucleic acid[8,9] With this information at hand, an accounting of the low efficiency of killing was made in terms of the Watson Crick model of DNA Any P^{32} disintegration it was though[illegible] cut that polynucleotide chain in which it occurred, for the recoil of the [illegible] nucleus should be sufficiently energetic to break phosphate [illegible] As the cutting of just one strand of the double helix would le[illegible] molecule intact and presumably functional, it was proposed that [illegible] gration can be lethal only if it happens to cleave also the second p[illegible] chain, thus leaving 2 double stranded fragments that could not [illegible] of the phage in reproductive processes Thus, under this scheme [illegible] *disintegrations that destroy the viability of the larger phage are [illegible]* only those that cleave DNA molecules

The correctness of this model seems assured in the light of m[illegible] studies into the effects of P^{32} decay within phage particles Thom[illegible] able to show that the efficiency of fragmentation by P^{32} decay of the [illegible] double-stranded DNA structure that is released from T2 particles by [illegible] shock or other means approximates the efficiency of inactivation of T2 particle as infectious units Despite this direct confirmation of the mechanism of action of P^{32} decay, one might wish to reserve judgment in view of the exceptional behavior of the very small phages S13 and ϕX 174, in which any P^{32} disintegration can cause death It is therefore of the utmost significance that the DNA of ϕX 174 appears on the basis of physicochemical studies to exist in a unique, possibly single-stranded, configuration[12] It would seem, in other words, that with these small phages additional evidence is provided of the validity of the model that Stent has proposed in explanation of the lethality of P^{32} decay in the T type and other phages

On turning to the lethal action of UV, it becomes necessary to take into account certain factors that are of no consequence in phage inactivation by P^{32} decay This is already made evident by the finding that UV survival curves in the conventional plot of the logarithm of the surviving fraction of phage particles versus the dose of UV are not always straight lines With some phages, for example T1 and P22,[4,13] the curves are concave upward, while with λ[14,15] and to a lesser degree with the T-even phages[13] they have a downward concavity Because of the complexity of the survival curves of certain phages it is rather difficult to compare the UV sensitivities of different strains, yet it is clear that their sensitivities to UV, unlike their sensitivities to P^{32} decay, do not vary according to the amount of DNA per particle[16]

The UV action spectra of bacteriophages[17,18] indicate that the loss of viability of phages on irradiation is caused primarily by photons absorbed by the nucleic

acid of the particles With T2 the quantum yield is reported[19] to be about 10^{-4} but in view of the failure to observe a correlation of UV sensitivity with DNA content on comparison of different phage strains it would appear that the quantum yields must vary considerably among phages in which the efficiencies of killing by P^{32} decay are the same That phage particles should differ in this respect is perhaps not surprising since the nucleic acids of at least some strains can be distinguished simply on the basis of their purine and pyrimidine base composition [9, 20-23] and the bases, which account for the UV absorbency of DNA, are not equally susceptible to chemical alteration under the influence of the radiation [24] While it is not yet possible to know to what extent the frequency of appearance of lethal lesions may be determined by the particular chemical constitution of the nucleic acid it is evident that the intrinsic UV sensitivity of a phage strain is a measure not only of the efficiency with which lesions are produced, but also of the frequency with which potentially lethal lesions are rendered ineffectual because they can be erased or circumvented on infection of the host bacterium Thus we have photoreactivation which can be demonstrated to a greater or lesser degree, depending on the phage strain, only after infection[25] and would appear in view of studies with transforming principle [26] to be mediated by an enzyme system of the host Recent evidence indicates that one or more other restorative or circumventive processes involve an interaction of phage and host structures in terms of which it is possible to account, at least in part, for the particular inactivation kinetics and UV sensitivities that have been found with various phage strains [4, 16, 27]

The lesions produced on UV irradiation whatever their nature must not cause any appreciable degradation of the phage nucleic acid This might be inferred from observations of the effects of UV on ribonucleic acid from tobacco mosaic virus[28] and is also suggested by the finding that UV given in the amount of many lethal doses does not suppress the ability of phage particles to inject a normal complement of DNA into host cells [29] Other evidence on this point comes from a comparison of the effects of UV and P^{32} decay on gene transfer during conjugation between opposite mating types of *E coli* K12 [30, 31] Transfer of genetic material in the K12 mating system is a unidirectional and oriented process in which markers of a donor-cell type pass to a recipient cell in a particular sequence that is indicative of their linear arrangement on the K12 linkage group In genetic recombination studies with donor cells highly labeled with P^{32} it has been found that the sensitivity to P^{32} decay of a given marker depends upon the distance of that marker from the leading end of the linkage group in transfer, provided that the radioactive decay occurs before mating In UV inactivation studies on the other hand different genetic markers of the donor appear equally sensitive to irradiation whether it occurs prior to or after transfer It is thus shown that the K12 chromosome suffers fragmentation on P^{32} decay but retains its structural continuity following UV irradiation

Inactivation of Intracellular Phage

UV irradiation and P^{32} decay have been employed as analytic tools in inquiries into the nature and growth of intracellular phage Much of the work

that has gone into such studies has been expended on one system phage T2 and its host *E coli* B, and it is to this system that comment will be confined here, although it should be borne in mind that investigations have been carried out with other systems upon aspects of phage reproduction other than those mentioned in the discussion In a number of respects, the T2 *coli* B system radiobiological study, it has been subjected to intensive investigation and happens to be one in which the host phage growth even after exposure to massive doses of UV latter point can become a rather important consideration to grasp the significance of the radiobiological properties exhibited by infected bacteria

The normal outcome of infection liberation of several years a tween infection of sensitivity t during the first minutes after are approximately as sensitive to UV as free T2 particles come progressively less sensitive and attain a top level of UV midway through the latent period The principle of the ment has been adopted by Stent in an examination of the of infection of P^{32} decay that occurs at different phage ...

... either nonradioactive cultures of *E coli* or cultures phage, are heavily labeled with the isotope As shown material whether growth of the radioactive parental active or ... which a become ... resistant and are already refractory to radioactive decay before the appearance of the first intracellular progeny particle

What interpretation might be given of these findings? The resemblance of the UV and P^{32} sensitivities of phage particles and early complexes can be attributed to the fact that both entities harbor a common radio sensitive substance This substance must be the DNA of the infecting phage particle that enters the host at the time of infection while the rest of the particle with the exception of some minor components, is left behind[36,38] Synthesis of phage DNA within the infected bacterium begins within a few minutes after infection[39] On close examination of the time course of the evolution of UV and P^{32} resistance, it is found[40] that DNA synthesis and the development of radio resistance commence at very nearly the same time Hence it might be posed that the ... simply a proceeds, DNA if a ever, a st

curves with time after infection is one of slope reduction rather than of increase in target number

Stent[35] has suggested that the evolution of the radioresistance of the complexes might be explained in terms of one of the following hypotheses

(1) Damaged replicas of the parental phage DNA can cooperate to produce one that is healthy

(2) The DNA of vegetative phage is intrinsically less sensitive to the lethal actions of UV and P^{32} decay than the DNA of the infecting phage particle

(3) The genetic attributes of the DNA of the infecting particle[4] are bestowed soon after infection upon some highly UV and P^{32} resistant substance that is not phage DNA

It is supposed under the first of these hypotheses that the DNA of vegetative phage can participate in a process similar to multiplicity reactivation which occurs in bacteria multiply infected with UV inactivated phage particles[42] but has not yet been demonstrated with particles inactivated by P^{32} decay Under the last two hypotheses the question of a possible role for protein has come to the fore in attempts to visualize what might take place during the evolution of radioresistance in the phage infected bacteria[16,35] Thus if it ... here

structural integrity of the unit as a whole If transfer of information occurred as required under the third hypothesis from phage DNA to a protein or nucleoprotein the complexes could certainly be expected to become highly UV resistant and refractory to P^{32} decay on completion of the process

Biochemical investigations with T2 infected *E coli* have demonstrated synthesis in newly infected cells of protein that is associated specifically with phage development Of several lines of evidence that can be offered in support of the view that this early protein is phage specific,[44] the most compelling is the fact that synthesis of phage DNA does not occur when infection takes place under cultural conditions that prevent protein synthesis[45-48] Thus on addition of chloramphenicol before or immediately after infection not only protein synthesis but also synthesis of phage DNA is prevented As the time lapse between infection and addition of chloramphenicol increase the effect of the antibiotic on DNA synthesis becomes progressively less pronounced and is no longer in evidence at a time when DNA synthesis is already under way[48,49] By that time also the buildup of the early protein is nearing completion[44] Enzymes concerned with the initiation of phage growth appear to be represented in this early protein[50] but the question at the moment is whether there might also be included some proteinaceous material* that plays a very direct role in the evolution of radioresistance in T2 infected bacteria

* ...res of the ...erial ... he RNA

The first point that might be made in this regard is that whether chloramphenicol is present on infection with T2 or is added at some later time when some decrease of UV resistance has already occurred the effect of the antibiotic is still the same, in the sense that it prevents the complex from becoming any more UV resistant[48] In other words, evolution of resistance to the maximum level is not achieved even in complexes in which synthesis of phage DNA, but not of protein, is permitted Uchida and Stent (personal communication) have tried to assess the radiobiological significance of the early protein by the execution of an experiment in which measurements are made at [illegible] various times after infection of the P^{32} sensitivity of genetic markers of [illegible] active T2 particles added along with chloramphenicol to [illegible] culture of *E coli* that was infected only a few minutes earlier [illegible] active T2 particles of another genotype Under this experi[illegible] where the only early protein present is that synthesized [illegible] between the primary infection and the superinfection it [illegible] genetic markers of the superinfecting phage remain sensitive [illegible]

It can be concluded on the basis of these findings that [illegible] involved in the establishment of the radioresistant state [illegible] matter however, to know how it is involved The result [illegible] carried out by Uchida and Stent implies that at least some of [illegible] formed under the direction of a T2 particle of one genotype [illegible] phage of another genotype This could mean that the [illegible] only in the reproduction of phage genomes identical to the [illegible] appearance, just as could be expected under the transfer [illegible] hypothesis On the other hand, it is possible that the uniqueness [illegible] the early protein has nothing to do with a genetic type of [illegible] not excluded that the result might be explained simply in terms of an [illegible]sibility of some early product of phage infection to any other incoming phage One might thus be led to suppose that the early protein has a role in the development of radioresistance only because it is required for DNA synthesis and that consideration should be given to the possibility that multiplicity reactivation might be the key to an understanding of the evolution of radioresistance in T2-*coli* B complexes

One would expect, on turning to the multiplicity reactivation hypothesis that the probability of reactivation should become greater and that the level of radioresistance should increase as the amount of phage DNA per bacterium increases Tomizawa and Sunakawa[48] have shown, however, that UV resistance does not evolve in complexes in which DNA accumulates in the presence of chloramphenicol To reconcile this result and that expected in terms of the hypothesis of multiplicity reactivation one must suppose that the reactivation process is impaired in complexes that have been treated with chloramphenicol As Tomizawa[56] has presented evidence to show that UV lesions within T2 DNA synthesized in the presence of chloramphenicol are neither repaired nor replicated prior to incorporation of the irradiated nucleic acid into mature particles, this supposition may have some merit but for the time being, one is left without an explanation of the evolution of radioresistance in the T2-infected bacterium, at least one that is supported by adequate proof

Conclusion

Although studies of virus development from a radiobiological point of view have been confined almost exclusively to investigations of bacteriophage growth, this monopoly may be short lived, for there is some justification[12] for the view that radiobiological techniques will find extensive use in studies of other viruses. It is to be hoped that information gained in bacteriophage studies will find useful application there.

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IONIZING RADIATION AND ITS EFFECTS ON ANIMAL VIRUSES*

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Whenever a new virus is isolated and it becomes possible to produce it in reasonable yield and to carry out assays with reasonable accuracy and convenience one immediately becomes curious about its physical characteristics such as size shape and internal structure in the hope that this knowledge will help in our understanding of its biological properties and also its relationship to other viruses Great progress in the physical characterization of viruses has been made in the last decade with the widespread adoption of tissue culture techniques for virus cultivation and assay, on the one hand and with the common availability of such physical tools as the analytical ultracentrifuge electron microscope, and radioisotopes on the other The recent use of ion exchange chromatography for the purification and fractionation of animal viruses has also greatly stimulated biophysical and biochemical studies

Ionizing radiation is of unique value in the physical study of animal viruses because neither high purity nor large quantities of virus are essential Furthermore and perhaps more significantly, inactivation by ionization gives information about size and shape of virus components in association with their biological activities The application of ionizing radiation studies to calculations of the dimensions of virus components has been presented in detail by Lea (1947) and by Pollard (1953 1955 1957) In their classic work published almost twenty years ago Lea and Salaman (1942) laid the foundations of the application of ionizing radiation to biological system particularly viruses establishing first that inactivation follows an exponential curve and second that 1 ionization is usually sufficient to inactivate 1 biologically active unit Furthermore it was clearly shown that the slope of the inactivation curve was a measure of the volume of the unit involved and that in fact at the ... volume ... In a ... inacti-

vation of vaccinia virus with X rays

Since the work of Lea and Salaman in the early 1940s on vaccinia virus and extensive studies on papilloma and fibroma virus by Friedewald and Anderson (1941 1943) and by Syverton *et al* (1941), published work on the effects of ... years ... ents ... th ... to ...

... the e ... levels of sophistication ... g vaccines or for sterilz...

* The work reported in this paper was supported in part by Research Grant E 2213 from the National Institute of Allergy and Infectious Diseases Public Health Service Bethesda Md

ing material suspected to be contaminated with viruses (2 [r ,re n [rt l inactivation accompanied by measurements of various l l v l l (3) nactivation of biological function with simultaneous [l y hen l]

treatment either with phenol or ultraviolet radiation Fr il (1) l Total inactivation doses for poliovirus Western equine e c[l t St I encephalitis and vaccinia have been recorded using gamma r tr n l

TABLE 1
RADIATION DATA ON ENTEROVIRUSE

Virus	Type of radiation	Property	Diameter (mμ)		
			Radiation	Other	
	Deuterons electrons	Infectivity	27	24*	Be s
ECHO 1	Alpha	Infectivity	36		B
	Electrons	CF antigen	13		Be
ECHO 7	Electrons alpha	Infectivity	30	24*	Ben h
	Electrons	CF antigen	8		Ben sh l l
	Deuterons alpha	Infectivity	30	28*	Pollard and K a t 19
Poliovirus	Electrons	Infectivity	30		Be esh l (1) 8
	Electrons	CF antigen	7		Benyesh *et al* (19 8)

* Ultrafiltration

In k r r — c n h h progressive

on a virus

TABLES 1 to 4 record the majority of published results on radiation inactivation on animal viruses together with unpublished data from our laboratory

size as estimated by such conventional methods as sedimentation ultrafiltration and electron microscopy

Enteroviruses

Initial experiments using deuterons and alpha particles to inactivate the MEF 1 — e a e a sensitive spherical unit of 1955) A recent extensive viruses Types 1

TABLE 2

RADIATION DATA ON INFLUENZA GROUP VIRUSES

Virus	Type of radiation	Property	Inactivation dose (r)	Diameter (mμ)		References
				Radiation	Other	
Influenza A	X ray	Infectivity		46	80 to 90†	
B	X ray	Infectivity		54		Buzzell *et al* (1955)
A	X ray	Infectivity	D_{37} 6.5 × 10^4	42		Buzzell *et al* (1955)
A	Deuterons electrons	Infectivity		42		Kroeger and Kempf (1959)
A	Alpha	Infectivity		76		Jagger and Pollard (1959)
A	Deuterons electrons	Hemagglutinin		12 × 2		Jagger and Pollard (1956)
B	Electrons	Hemagglutinin		MW 200 × 10^6	12.5–23	Jagger and Pollard (1956)
A	X ray	Hemagglutinin		MW 150 × 10^6		Woese (1953)
A	X ray	Hemagglutinin	D_{37} 3.3 × 10^5	7 S		Buzzell *et al* (1955)
A	Deuterons electrons, alpha	Interference		23 25 × 4		Kroeger and Kempf (1959) Powell and Pollard (1956)
A	Electrons	Infectivity	1.8 × 10^5*			Bellamy *et al* (1957)
Newcastle disease	Deuterons alpha electrons X ray	Infectivity		99	110†	Woese and Pollard (1954)
	Deuterons alpha protons	Infectivity		56		Wilson and Pollard (1958)
	Deuterons electrons	Hemagglutinin		8		Woese and Pollard (1954)
	Deuterons alpha protons	Hemolysin		24 × 2		Wilson (1958a)

* Total inactivation

† Electron microscope

and 7 also showed remarkably good agreement between the size of the infectious particle calculated from radiation inactivation experiments and the size of the entire virus particle as measured by ultrafiltration and electron microscopy (TABLE 1). The complement fixing (CF) antigens could be represented as spheres 13, 8, and 7 mμ in diameter for ECHO 1, ECHO 7, and poliovirus, respectively (Benyesh *et al.*, 1958).

Influenza and Newcastle Disease Viruses

These myxoviruses have recently been studied extensively with a wide variety of radiations (TABLE 2). Calculations of the size of the influenza infectious unit from X ray (Buzzell *et al.*, 1955; Kroeger and Kempf, 1959) deuteron and electron experiments (Jagger and Pollard, 1956) by various workers are in excellent agreement, giving a diameter of 42 to 54 mμ; the diameter from alpha particle irradiation is 76 mμ (Jagger and Pollard, 1956). Ion-density curves obtained by Jagger and Pollard also suggest the presence of an inner radiation-sensitive core 50 mμ in diameter, surrounded by a relatively insensitive shell 13 mμ thick, which correlates closely with the dimensions of structures seen in ultrathin sections of the virus (Morgan *et al.*, 1956). The interfering unit of influenza may be represented as a shell 25 mμ in diameter and with a molecular weight of 1.6 × 10⁶ (Powell and Pollard, 1956). Newcastle disease ... modern unit

The hemagglutinins of influenza and NDV, as with all viruses so far studied, are extremely resistant to radiation inactivation; the influenza hemagglutinins may be represented as a pair of spheres 7.5 mμ in diameter (Buzzell *et al.*, 1955) or as a pair of plates 12 mμ × 2 mμ (Jagger and Pollard, 1956). The molecular weight of such hemagglutinins would be between 150 × 10³ and 200 × 10³ (Jagger and Pollard, 1956; Woese, 1953). In a recent X ray study, inactivation of the hemagglutinin of influenza virus concentrated by ultracentrifugation followed a multiple-hit curve, apparently due to the aggregation of virus particles in the pellet; hemagglutinin released by disrupting the virus with ether was inactivated exponentially, giving a target diameter of 23 mμ (Kroeger and Kempf, 1959). These particles were said to be 12.5 mμ in diameter from electron micrographs (Hoyle *et al.*, 1953). The NDV hemagglutinin ha ...

each of mole ...

The hemol ...

we have any ...

there are about 15 hemolytic units distributed over the virus sur... in the form of plates 24 × 2 mμ (Wilson and Pollard, 1958) as illustrated in FIGURE 1. All of these units must be intact for maximum hemolysis rate, but any surviving unit can produce total hemolysis if given sufficient time (Wilson, 1958*b*).

Tumor Viruses, Measles and Herpes

TABLE 3 records radiation data for a miscellaneous group of medium-sized viruses including Shope papilloma, Rous sarcoma, and Shope fibroma. Two

extensive papers on X-ray irradiation of the papilloma ... 1941 In these the dose for total inactivation of a fre h ... from 4 to 6 × 10⁶ r, and the CF antigen was completely ... 10⁶ r (Friedewald and Anderson, 1941; Syverton *et al* 1941 ... by centrifugation was inactivated, however, by doses of ... it was concluded that the protein present in crude tum ... the virus against radiation (Friedewald and Anderson 1941 ... *incidental finding was that the yield of virus extracted fr* ... tumors increased up to one hundredfold The rabbit fibr ...

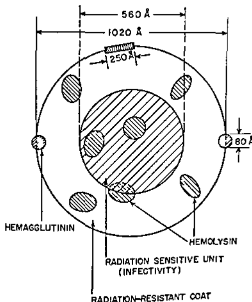


FIGURE 1 Schematic drawing of Newcastle disease virus (NDV) based on radiation data Reproduced from Wilson and Pollard (1958) with permission from *Radiation Research*

larger virus (200 × 240 mμ), was 90 per cent inactivated by a relatively small dose of the order of 10^4 r (Friedewald and Anderson, 1943) Extremely thorough X ray studies on Rous sarcoma in varying degrees of purification have also been made the best data were obtained with a "microsome" fraction after 2 cycles of differential centrifugation, giving a 37 per cent inactivation dose of 4 6 × 10^4 r, equivalent to a radiation sensitive core 46 to 49 mμ in diameter (Bryan *et al* 1950)

The infectious ... very recently wit ... (Moore *et al*, 1959) ... concluded that the agent ("milk factor") occurs in two forms, one 90 to 120 mμ, and the other 15 to 35 mμ in diameter, the latter representing an infectious "nucleoid" Milk that received small doses of irradiation was more infectious

than before, possibly because of the destruction of an inhibitor. Measles and herpes viruses have radiation sensitive infectious units 58 mμ (Benyesh *et al.*, 1958) and 41 to 52 mμ in diameter, respectively (Powell, personal communication).

Vaccinia

Let us now consider vaccinia, the largest animal virus thus far studied in detail with radiation techniques, and also the one used in the earliest recorded work of this type (Gowen and Lucas, 1939; Lea and Salaman, 1942). Although physicists for many reasons usually prefer to work with smaller and relatively less complex viruses, I have chosen vaccinia because its very complexity and multiple biological properties may be particularly revealing and rewarding in studies with ionizing radiation. The assays available for infectivity, hemagglutinin, and the antigens are relatively convenient and precise, and the virus itself is comparatively stable to high vacuum drying and other physical manipulations necessary for radiation work. An added point of interest is that vaccinia is a desoxyribonucleic acid (DNA) virus, and recombination between strains has recently been demonstrated (Fenner and Comben, 1958).

Low-Energy Electrons

These studies utilized a low voltage electron accelerator constructed by my colleague J. W. Preiss and used by him to localize enzymes within the yeast cell (Preiss, 1958). The unique feature of such an apparatus is that it employs electrons of known energies and, consequently, of known depths of penetration. These depths have been determined experimentally by bombarding dry enzymes in films of known thickness (Davis, 1954) and have also been calculated theoretically (Lea, 1947). The voltages used in our experiments varied from 500 (range approximately 100 Å) to 5000 (range approximately 6000 Å).

In experiments of this type it is important to use virus as pure as possible, since obviously a coating of salt or contaminating protein or lipid from the host cell will interfere with the penetration of such low-energy particles. Furthermore, the virus sample should be sufficiently dilute to ensure that the dried virus is only one particle thick. Unfortunately, although the virus withstands differential sedimentation and washing in distilled water and dialysis against distilled water with little or no loss in activity, the drying of such material on stainless steel gave extremely erratic results. It was therefore necessary to use virus simply dialyzed and diluted 10^{-2} to 10^{-3} in distilled water, which could then be lyophilized on stainless steel disks with very little loss of activity. As shown below, probably no more than 5 to 10 per cent of this material is shielded by foreign matter.

Results of such experiments, which were carried out jointly with J. W. Preiss, are shown in FIGURE 2. The assay in these experiments, as in all others unless stated to the contrary, was done by counting pocks on the chorioallantoic membrane of chick embryos inoculated by standard techniques. Exposure to 500 v did not reduce infectivity below that of the controls, which were placed in the chamber out of range of the electron beam. At 1000 v (penetration 300 Å) there was great scatter in the data, with some evidence of inactivation

at the highest dose (15 × 10^{13} electrons/sq cm) When 1 000 ... used, however, there was clearly a sudden drop in in... flattening at about 10 per cent of the control titer ... that ionizations occurring at 600 to 700 Å or deeper ... rapidly inactivate the infectious mechanism Other ... 2000, 2500, 4000, and 5000) caused similar rapid ina... at a persistent fraction of about 2 to 5 per cent, thi... represents virus shielded by foreign substances

One may tentatively conclude, therefore, that the ... at least 500 to 600 Å in thickness, which is not essent...

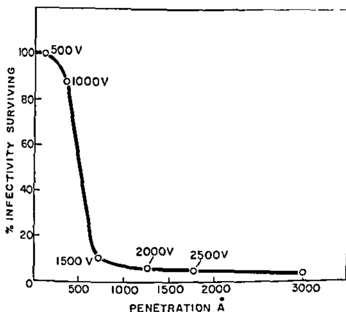


FIGURE 2 Inactivation of vaccinia virus infectivity by irradiation with slow electron penetrating to 3000 A

...ess or at least is not significantly affected by ionizations occurring within it The implications of these findings on the structure of the virus are discussed below In further experiments we intend to study slow electron inactivation of the hemagglutinin, antigens, and enzymatic activity of vaccinia and other viruses of the pox group

High Energy Particles

Exponential inactivation curves for both infectivity and hemagglutination are obtained when vaccinia is irradiated with high energy electrons from a 1 mev Van de Graaff accelerator or with gamma rays from a cobalt 60 source Data from one such experiment, shown in FIGURE 3, indicate exponential loss of infectivity down to about 1 per cent of the control titer The 37 per cent

inactivating dose of radiation (that is the point at ...

sol... (estimated) 300,000 r/hour Although particul... tention was given to standardizing the drying techniques and the irrad... was done at 0° C, the data show more scatter than those obtained by ... methods Nevertheless, the slope of the curves thus obtained indicates

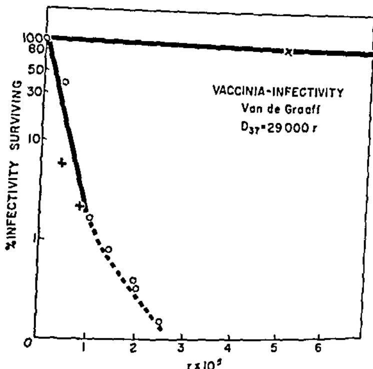


FIGURE 3 Inactivation of vaccinia virus infectivity by irradiation with fast electro... from a Van de Graaff accelerator Upper solid line represents inactivation of hemagglutin... on the same scale

per cent inactivating dose (D_{37}) of 40 000 r, equivalent to a sphere 46 mμ in diameter Again, excellent exponential curves are obtained down to roughly 5 per cent of the control titer, after which the curve flattens out for unexplained reasons (McCrea and O Loughlin, in preparation)

TABLE 4 summarizes the vaccinia infectivity data thus far obtained by various workers

The average D_{37} calculated by Lea and Salaman (1942) is larger than ours by a fac... when on... tions by... calculations of sensitive infectious unit diameter is, in fact, surprisingly good

Ionizing radiation is of particular value in studying virus components such as hemagglutinins, that only rarely can be completely separated in active stable form from the infectious unit. Most of the experiments described here were done before a complete separation of the vaccinia hemagglutinin was possible and hence gave the first clues to its small size (McCrea and Harris, in preparation).

[illegible]

ionizing radiations, the D_{37} being of the order of 4.0 to 5.0 × 10 r. Since most of the hemagglutinin is unattached to the virus, the volume may be calculated as a single sphere of diameter 90 to 100 Å. Many experiments on the virus *in vacuo*, using both fast electrons and gamma rays, have confirmed th[illegible]

TABLE 4
RADIATION DATA ON VACCINIA V[illegible]

Type of radiation	Property	Inactivation dose (r) D_{37}	Diameter mμ		
			Radiation	Oth[illegible]	
X ray	Infectivity	1×10^5	34	260 × 1[illegible]	[illegible]
Alpha	Infectivity	2×10^5	33		[illegible]
Gamma	Infectivity	8×10^4	36		[illegible]
Electrons gamma	Infectivity	4×10^4	46 to 50		
Slow electrons	Infectivity	—	70		[illegible]
Electrons gamma	Hemagglutinin	4.5×10^6	9 MW 350 000		[illegible]
X ray	Infectivity	4×10^6*	—		[illegible]
Gamma	Infectivity	2×10^6*	—		[illegible]

* Total inactivation
† Electron microscope

value. As FIGURE 4 shows, hemagglutinin irradiated when wet is considerably more *sensitive* to inactivation. This is to be *expected from its small size* which makes it particularly susceptible to the indirect effects of radicals formed in solution exposed to high energy ionizations.

Hemagglutinin 'purified' by removing the bulk of the infectious particles by differential centrifugation tends to aggregate and precipitate after standing for a few hours in the cold and, at the same time, it rapidly loses activity (Gillen *et al.*, 1950). These effects possibly explain why previous filtration and sedimentation experiments indicated that the hemagglutinin was 65 to 100 mμ in diameter (Chu, 1948). Recently we have been able to separate completely the hemagglutinin in a stable form from the infectious virus particles by chromatography on DEAE cellulose (McCrea and O'Loughlin, 1959). Sedimentation and other studies on this material give dimensions of similar order to those calculated above from radiation experiments.

The final picture of the vaccinia particle from radiation experiments thu[illegible]

inactivating dose of radiation (that is, the point at which, according to the Poisson distribution, 1 ionizing event per virus particle occurs) is about 30 000 r, which is equivalent to a sensitive volume of 6.7×10^{-17} cu cm, and hence to a spherical unit of approximately 50 mμ diameter

A large number of experiments has also been carried out using a cobalt 60 source that delivers approximately 300,000 r/hour Although particular attention was given to standardizing the drying techniques and the irradiation was done at 0° C, the data show more scatter than those obtained by other methods Nevertheless, the slope of the curves thus obtained indicates a 37

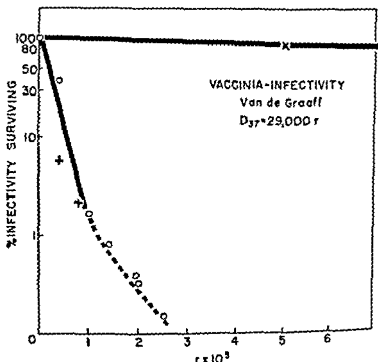


FIGURE 3 Inactivation of vaccinia virus infectivity by irradiation with fast electrons from a Van de Graaff accelerator Upper solid line represents inactivation of hemagglutinin on the same scale

per cent inactivating dose (D_{37}) of 40,000 r, equivalent to a sphere 46 mμ in diameter Again, excellent exponential curves are obtained down to roughly 5 per cent of the control titer, after which the curve flattens out for unexplained reasons (McCrea and O Loughlin, in preparation)

TABLE 4 summarizes the vaccinia infectivity data thus far obtained by various workers

The average D_{37} calculated by Lea and Salaman (1942) is larger than ours by a factor of approximately 3, but this difference is probably not significant when one considers that these investigators performed their infectivity titrations by the relatively inaccurate assay in rabbit skin The agreement in the calculations of sensitive infectious unit diameter is, in fact, surprisingly good

Ionizing radiation is of particular value in studying virus components, such as hemagglutinins, that only rarely can be completely separated in active stable form from the infectious unit. Most of the experiments described here were done before a complete separation of the vaccinia hemagglutinin was possible, and hence gave the first clues to its small size (McCrea and Harris, in preparation).

FIGURE 4 gives examples of the exponential curves obtained from irradiating the hemagglutinin with 1 mev electrons in the wet or dry state. The curve for dry virus shows the high resistance of the hemagglutinin to inactivation by ionizing radiations, the D_{37} being of the order of 4.0 to 5.0 × 10^6 r. Since most of the hemagglutinin is unattached to the virus, the volume may be calculated as a single sphere of diameter 90 to 100 Å. Many experiments on the virus *in vacuo*, using both fast electrons and gamma rays, have confirmed this

TABLE 4
RADIATION DATA ON VACCINIA VIRUS

Type of radiation	Property	Inactivation dose, r, D_{37}	Diameter, mμ: Radiation	Diameter, mμ: Other	Reference
X ray	Infectivity	1 × 10^5	34	260 × 210†	Lea and Salaman (1942)
Alpha	Infectivity	2 × 10^6	33		Lea and Salaman (1942)
Gamma	Infectivity	8 × 10^4	36		Lea and Salaman (1942)
Electrons, gamma	Infectivity	4 × 10^4	46 to 50		McCrea (unpublished)
Slow electrons	Infectivity	—	70		McCrea and Preiss (in preparation)
Electrons, gamma	Hemagglutinin	4.5 × 10^6	9, MW 350 000		McCrea and Harris (in preparation)
X ray	Infectivity	4 × 10^4*	—		Gowen and Lucas (1939)
Gamma	Infectivity	2 × 10^6*	—		Jordan and Kempe (1956)

* Total inactivation
† Electron microscope

value. As FIGURE 4 shows, hemagglutinin irradiated when wet is considerably more sensitive to inactivation. This is to be expected from its small size, which makes it particularly susceptible to the "indirect" effects of radicals formed in solution exposed to high-energy ionizations.

Hemagglutinin purified by removing the bulk of the infectious particles by differential centrifugation tends to aggregate and precipitate after standing for a few hours in the cold and at the same time it rapidly loses activity (Gillen *et al.*, 1950). These effects possibly explain why previous filtration and sedimentation experiments indicated that the hemagglutinin was 65 to 100 mμ in diameter (Chu, 1948). Recently we have been able to separate completely the hemagglutinin in a stable form from the infectious virus particles by chromatography on DEAE cellulose (McCrea and O'Loughlin, 1959). Sedimentation and other studies on this material give dimensions of similar order to those calculated above from radiation experiments.

The final picture of the vaccinia particle from radiation experiments thus

suggests a ra l at on en r
sphere locatc
diameter anc
Slow electron
below which u o en on causes a rapid drop in infectivity The differenc between these two sets of data is probably not significant when one consider possible variations in calibration of slow electron ranges and also the varia tions due to drying resuspending and titrating an animal virus That th internal structure of vaccinia is extremely complex has been clearly demon

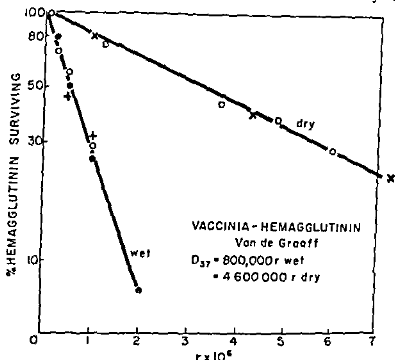


FIGURE 4 Inact vation of vacc nia hemagglutinin by irra l ation with fast electrons from a Van de Graaff accelerator

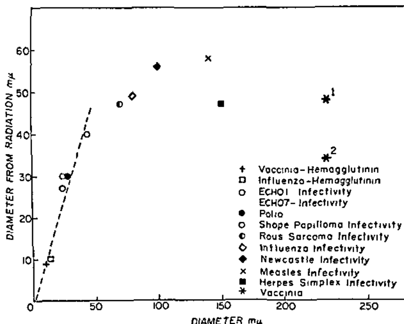
strated in recent electron micrographs of thin sections of the virus by Epstein (1958) and with further radiation studies it may be possible to correlate func tional units with certain of these structures seen in sections

The third type of radiation study mentioned in the introduction namely correlation of radiation action with physical or chemical changes in the virus particle has been scarcely applied to animal viruses Well marked changes in the vi cosity of ribonucleic acid (RNA) from tobacco mosaic virus show that breaks in the nucleic acid threads occur after treatment with X rays (Lauffer *et al* 1956) and it would seem worthwhile to extend this type of study to animal viruses At present we are carrying out preliminary experiments to detect physical changes in vaccinia irradiated with electrons electron micrographs of sectioned virus show severe disorganization of internal structure after

exposure to 300,000 r, as compared to unirradiated controls, but further work is needed to establish the nature and specificity of these changes

Conclusions

After an analysis of the radiation data on animal viruses, one may perhaps attempt a brief synthesis. In all, 10 viruses have been irradiated in a manner that allows accurate calculations of the dimensions of functional units to be made from target theory. An interesting relationship between the radio



[illegible] h rizontal scale

sensitive dimensions and the total virus dimensions is revealed when these are plotted, as shown in FIGURE 5. There is an excellent linear relation between the diameter of a unit calculated from target theory and by other methods up to a total virus diameter of approximately 45 mμ. For viruses below this size, therefore the whole particle corresponds closely to the radiosensitive volume. Lea (1947) has pointed out that the volume of small bacteriophages agrees closely with that calculated from radiation data whereas, with large viruses such as vaccinia, the radiosensitive infectious volume represents less than 1 per cent of the total volume (Lea and Salaman, 1942). Epstein (1953) showed that there was excellent agreement between radiosensitive volume and nucleic

acid volume in several plant viruses and T7 bacteriophage As FIGURE shows, however, for animal viruses larger than 45 mμ, *the diameter of the radia tion sensitive infectious unit remains approximately constant irrespective of th size of the entire virus* Thus all viruses larger than 45 mμ in diameter so fa examined including

and vaccinia sho

and all but NDV

periments on other viruses are needed, of course, but from the available dat it appears that for viruses above 45 mμ in diameter the average radiosensitive infectious unit is 49 mμ in diameter, corresponding to a spherical volume of 6×10^{-17} cu cm and a molecular weight of 45 million Further work will be necessary to establish the validity of these values and to show their relation ship, if any to the total nucleic acid content of the larger animal viruses

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EFFECTS OF PHYSICAL ENVIRONMENT ON THE VIRUS OF FOOT-AND-MOUTH DISEASE*

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Foot and mouth disease is classified among the highly contagious diseases of cloven hoofed animals Although it remains a scourge in various parts of the world it is of no importance as a disease problem in the United States at the present time The last outbreak occurred three decades ago in California Cattle in our northern and southern neighbors, Canada and Mexico, have had the disease since that time Through the prescribed eradication measures of slaughter of diseased and exposed animals, disinfection of contaminated premises, and vaccination of other cattle, the disease eventually was eliminated from these areas

The highly contagious nature of foot and mouth disease makes it imperative that the virus be managed with regard to strict safety measures in laboratories [illegible] consideration also must [illegible] ducts [illegible] gicals Concurrently, regulatory agencies of the government must prescribe definitive means of controlling importation, processing, inactivation of this virus and other exotic disease agents, and disposal of wastes from animal materials that originate from areas of the world in which foot and mouth disease is enzootic

By virtue of its extreme communicable predisposition, methods must be made available for inactivating the virus both in the laboratory and in the field It is highly desirable to make use of noninfectious antigens when performing serologic tests This, at least, would eliminate one pathway of exit of live virus from the laboratory to a susceptible animal population In the field, adequate simple, and inexpensive methods are necessary for disinfecting areas in which foot and mouth disease is present In this case chemical treatment of virus contaminated materials is more feasible A review of chemical inactivation studies of foot and mouth disease virus is presented elsewhere in this monograph

Reports on the effects of physical agents on the virus of foot and mouth disease have been far less numerous than for most other infectious agents In the United States, governmental regulations have not permitted work with the virus within its boundaries in recent years Results on inactivation studies began to appear from Europe in 1897 These and the few scattered reports that were made in subsequent years were accepted until recently With the opening of the governmental research facilities at the Plum Island Animal Disease Laboratory, United States Department of Agriculture, Greenport, N Y, several interesting and important findings have been brought to light concerning the effects of heat on the virus

[illegible] introduction to a survey of the literature on the subject of inactivation

of foot and mouth disease virus, it was clearly evident that many experimental details were omitted in the published reports In many cases, especially in the initial publications, such particulars as virus source and history type and number of test animals, route of inoculation volume of inoculum, type and pH of the suspending medium, dilution of the initial virus preparation, titer of the inoculum, and the exact mechanics of the inactivating procedure were not given It must be remembered, of course, that in the early work some of the above information was not available since the science of virology was in its infancy

The survey that follows is given to point out in part, the inadequacy of the information that is accessible and also to give data on the more recent findings with respect to the treatment of the virus with physical agents for the purpose of inactivation Problems and methods of determining the criteria for loss of infectivity also will be considered Major emphasis will be given to the latest available data that were obtained during studies of heat inactivation of foot and mouth disease virus during the last five years principally from the Plum Island Animal Disease Laboratory

Effects of Nonionizing Radiation and Ultrasonic Energy

In studies undertaken to determine the immunogenicity of virus exposed to ultraviolet irradiation vesicular fluids from guinea pigs and cattle experimentally infected with homologous Type-A Vallee foot and mouth disease virus were used as prospective sources of the vaccines [1,2] No information was cited concerning the energy and the wave length used although mention was made of the fact that irradiation took place in the ultrashort range of the ultraviolet band Rates of flow through the irradiation chamber and exposure periods were given

Several methods for the preparation of the materials were employed In the first series of experiments the infectious fluids were mixed and adsorbed on aluminum hydroxide diluted with water and subsequently exposed to irradiation

By testing guinea pig virus in guinea pigs it was found that an exposure time of more than 65 sec would destroy infectivity whereas immunogenicity was retained for as long as 540 sec of exposure Details on the procedures for challenging with active virus in order to determine the possible immunogenic properties of the vaccine were not given

When bovine virus was tested in guinea pigs the observation was made that periods of exposure to irradiation of less than 45 sec were ineffective in destroying the infectious property for guinea pigs The treated material did not produce signs of foot and mouth disease when exposed for 165 sec and engendered a satisfactory immune response as determined by the inoculation of active virus Exposure of the virus for 60 sec did not completely destroy the infectivity However at 300 and 400 sec of irradiation 50 per cent of the guinea pigs inoculated with treated material developed signs of the disease No explanation of this phenomenon was given

Using bovine virus material and testing innocuousness in cattle after exposure of the virus for as long as 165 sec it was found that in most instances infectivity could be destroyed

In the second series of experiments in which native infected fluids were diluted with physiological saline and exposed, it was found that longer irradiation times were necessary to inactivate the infectious property of the virus in fluids containing proportionally more virus

It was emphasized that, in the testing for innocuousness of vaccines, the materials be tested in the species from which they were derived This suggestion was made because, when adapted virus was inoculated into a heterologous host, a lower infectious titer was obtained Consequently, the ability of the foreign host to detect small amounts of active virus in a treated vaccine was decreased

Studies on the effects of ultrason ... re reported[3] An ... deleterious effect

Effects of pH

Native infected animal tissues and products The British have been prolific with work on the survival of foot and mouth disease virus in meat and other animal products[4] In studies made with beef carcasses and organs it was found that the persistence of bovine adapted Types A and O viruses, as tested in cattle, was closely related to the *p*H of the tissues Similar conclusions were arrived at by German workers when experimenting with guinea pigs[5] Acid produced in muscle during the process of *rigor mortis* rapidly inactivated the agent, although by rapid freezing the formation of acid could be suspended, consequently preserving the virus When quick frozen meat was thawed, the process of acid production was initiated, and the infectivity of the agent was destroyed

Production of acid in liver, kidney, rumen, lymph node, and blood was not as rapid as it was in muscle tissue, and prolonged survival was more likely even when there was a delay in freezing and after thawing[4]

In previous work on a similar type of study, swine were used in attempts to detect bovine virus in bovine offal[6] It was concluded from the negative results obtained that host adaptation and sensitivity of the host to heterologous virus strains were highly important factors in the determination of infectivity of foot and mouth disease virus As further supportive evidence for this conclusion, when guinea pig adapted virus in guinea pig offal was tested in guinea pigs it was found that infectivity could be detected

Tissue culture fluids, vesicular fluids, and tissue extracts The successful cultivation of foot and mouth disease virus, Type A, strain 119, in tissue culture[7] has facilitated procedures in the determination of the physical and biological properties of the agent

In studies designed to measure the *p*H stability of the virus, fluids from infected tissue cultures of bovine kidney epithelial cells were used[8] The virus was stored in buffers of various *p*H values at 4° C and tested after appropriate intervals of time in tissue cultures The original infective titer was maintained for at least the 5 week observation period when the virus was stored at *p*H 7 0 to 7 9 A 90 per cent reduction in infective titer occurred each interval of 1 min , 1 min , 14 hours, 3 weeks, 1 week, and 14 hours at *p*Hs 5 0 6 0, 6 5, 8 0, 9 0, and 10 0, respectively

Extending these studies further, it was determined by tissue culture methods and using tissue culture adapted virus that a minute fraction of the virus population was highly resistant to changes in *p*H At *p*H 5 0 one one millionth of the original virus population was found to be resistant to further inactivation for approximately 30 min, while at *p*H 6 0 one one millionth of the virus persisted for at least 60 min

It is not known at the present time whether this resistant fraction is related to environmental conditions or to genetic differences If the observations

dermally into the metatarsal pads it was found that when the virus was stored at 37° C in buffers of various *p*H values it would not have the ability to infect after 5 days at *p*H 6 9 and after 3 days at *p*H 8 0, whereas it produced active infection after 7 days at *p*H 7 9 and after 9 days at pH 7 6

Effects of Desiccation

The results of one study concerning a change in immunological type subsequent to drying have appeared[10] Dry bovine Type O virus was inoculated into guinea pigs and vesiculation and healing were allowed to follow their courses When the guinea pigs were challenged with native bovine Type A virus, signs of infection were not apparent It is known that there is no significant cross immunity with experimental infections of foot and mouth disease in the guinea pig The hypothesis was presented that desiccation caused a change in the type virus by a process of mutation No mention was made of the possibility that a heterologous virus population may have been present in the original inoculum Considering experimental conditions this would have to be presented as a strong possibility

Effects of Heat

Considering the limited amount of experimental work that has been reported on inactivation studies of foot and mouth disease virus by physical methods the data comprising results of investigations on the effects of heat on the antigenicity and infectivity of the virus have been more voluminous and of more usefulness and importance than the data accumulated using other methods of inactivation Although much of the information was not detailed especially in the initial studies a general trend was observed There was considerable lack of confirmation of experimental results The probable reasons for lack of agreement will be enumerated during the presentation of these findings that follow It must be remembered that due to the scanty experimental details and methods used in the primary reports, much of the meaning of the results is now thought to be outdated in light of present-day interpretations The probable causes will also be discussed

The results of the effects of heat on various materials containing foot and mouth disease virus would be summarized best in tabular form and are given

Table 1
Effects of Heat on the Survival of Foot-and-Mouth Disease Virus in Blood

Reference number	Virus history	Medium	Temperature and time	Route of inoculation and test animal	Results (infectivity)
11	Unidentified	Not mentioned	37° C 82 days	Not mentioned	Active
12	Unidentified	Not mentioned	80–100° C short time 60– 0° C 25 min	Not mentioned Not mentioned	Inactive Inactive
16	Unidentified	Not mentioned	50° C 1/2 min	Not mentioned	Inactive
17	Unidentified	Not mentioned	55° C 15 min 55° C 20 min	Not mentioned Not mentioned	Active Inactive
11 14	Guinea pig	None	25° C 19 days 37.5° C 24 hours 42° C 12 hours	Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig	Active Active Active
9	Guinea pig	Not mentioned	37° C 24 hours 37° C 48 hours	Intradermal guinea pig Intradermal guinea pig	Active Inactive
17	Guinea pig	Not mentioned	50° C 2 1/2 hours 55° C 20 minutes 60° C 2 minutes	Not mentioned Not mentioned Not mentioned	Active Inactive Inactive
13	Guinea pig	Not mentioned	37° C 24 hours 37°C 48 hours 37°C 3 days	Intradermal guinea pig Intradermal guinea pig Not mentioned	Active Inactive Inactive
16	Guinea pig	Not mentioned	50° C 4 hours 50° C 4 2/5 hours 51° C 2 1/2 hours 51° C 4 2/5 hours 53° C 40 min 53° C 1 hour 55° C 15 min 55° C 20 min 60° C 2 min	Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig Intradermal guinea pig	Active Inactive Active Inactive Active Inactive Active Inactive Inactive

13	Defibrinated guinea pig Types A O C	1 10 phosphate buffer pH 7 6	37° C 21 days	Intradermal guinea pig	Inactive
	Citrated guinea pig Types A O C	1 10 phosphate buffer pH 7 6	37° C 49 days	Intradermal guinea pig	Inactive
11	Bovine	Not mentioned	37 5°C 5 days 41° C 12 hours 55° C 15 min 60° C 15 min. 70° C 15 min	Not mentioned Not mentioned Not mentioned Not mentioned Not mentioned	Active Active Active Active Active
15	Bovine	None	60° C 15 min 58° C 15 min	Cattle Cattle	Active Active
16	Bovine	Not mentioned	55–70° C	Not mentioned	Not completely inactivated

that many particulars describing experimental conditions were missing in the reports that are cited. There were times when the source of the blood, the suspending medium, the test species, and route of inoculation were not given. Without knowledge of these details it is difficult to stipulate conditions that are necessary for the total destruction of infectivity in blood containing foot and mouth disease virus. *Some of the divergences in results that were obtained* may have been due to differences in susceptibility of various test animals, routes of inoculation, virus titers, and virus types. The data do indicate that conditions for destroying the infectious property of the virus in bovine and guinea pig blood are different. Generally, a more drastic environment for a longer period of time is needed for bovine blood. The reasons for these differences are not known definitely. Again, we must remember that all of the *experimental details were not known in most of these cases.*

TABLE 2
EFFECTS OF HEAT ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS IN NATIVE ANIMAL TISSUES

Reference number	Virus history	Medium	Temperature and time	Route of inoculation and test animal	Results (infectivity)
11	Guinea pig vesicle coverings	Not mentioned	Not mentioned 54–63° C, 4 days	Not mentioned Not mentioned	Inactive Inactive
19	Guinea pig vesicle coverings	50% glycerol phosphate buffer *p*H 7.5	37° C, 5 days	Not mentioned	Active
		Phosphate buffer *p*H 7.5	37° C, 5 days	Not mentioned	Inactive
20	Guinea pig sweetbreads Type O	Water	1.22 min at 60° C or over	Intradermal guinea pig	Inactive

Native animal tissues. The results of the effects of heat on the survival of foot and mouth disease virus in native animal tissues are given in TABLE 2. Evidently the data are too few to reach any conclusions. There is one indication that glycerol enhances the survival of the virus under the *conditions of* the experiments.

Body secretions and excretions. The results of limited studies of the effects *of heat on the survival of foot and mouth disease virus in milk, saliva and manure are given in TABLE 3. Conclusions* on this number of data are not warranted.

Vesicular fluids. The results of varied experiments on the effects of heat on foot and mouth disease virus present in vesicular fluids *are given in* TABLE 4. There are wide variations in the results obtained. Factors possibly responsible for the differences will be discussed later.

Desiccated materials. A summary of results of the effects of heat on the survival of foot and mouth disease virus in desiccated materials is given in TABLE 5. Comparing these results with the others presented in TABLES 1 through 4, it is apparent that lack of moisture increases the resistance of the virus to heat.

Tissue culture fluids One report has appeared in the literature[8] on the effects of heat on the survival of the virus in fluids from cultures of bovine kidney epithelial cells infected with Type A, strain 119 virus When stored in Veronal acetate buffer at *p*H 7 5 and heated at various temperatures and periods of time, it was found that 90 per cent of the virus would be inactivated as follows 61° C, 3 sec to a survival of 0 00001, 11 min thereafter 55° C, 20 sec to a survival of 0 001, 7 min thereafter, 49° C, 1 hour 43° C, 7 hours, 37° C, 21 hours, 20° C, 11 days, and 4° C, 18 weeks

A minute fraction of the virus was found to be highly resistant to inactivation by heating at 55° and 61° C This resistance tends to raise questions concerning the possible homogeneity of the virus when grown in tissue culture

The loss of infectivity was found to proceed by 2 different methods, as calculated from activation energies The Arrhenius plot gave 2 straight lines that joined at 43° C Activation energies calculated from the slopes of the lines below 43° C were 27,200 calories per mole whereas, above 43° C 120 600

TABLE 3
EFFECTS OF HEAT ON THE SURVIVAL OF FOOT AND MOUTH DISEASE VIRUS IN SECRETIONS AND EXCRETIONS

Reference number	Virus history	Medium	Temperature and time	Route of inoculation and test animal	Results infectivity
12	Milk	Not mentioned	80-100° C short time	Not mentioned	Inactive
			60-64° C 30 min	Not mentioned	Inactive
			70° C 15 min	Not mentioned	Inactive
21	Saliva and manure	None	53° and 70° C 3 6 and 9 days	Intravenously and scarification on oral mucosae cattle	Inactive

calories were needed to destroy the infectivity of foot and mouth disease virus when adapted to tissue cultures of bovine kidney

Unidentified virus sources Since the results given in TABLE 6 are not detailed sufficiently conclusions concerning the inactivation of foot and mouth disease virus in these unidentified sources cannot be given with any degree of accuracy The effects of heat on the complement fixing activity of the antigen are mentioned in 2 instances[25,26] In these cases exposure times of 56° C for as long as 1 hour and 60° C for as long as one half hour did not alter the serologic activity significantly whereas infectivity of the virus was not always destroyed More detailed experiments on the effects of heat on the infectivity and antigenicity of foot and mouth disease virus will be given in the discussion on the effects of heat on virus in tissue extracts

Tissue extracts Summarized results on the effects of heat on the virus are given in TABLE 7 Only data reported prior to 1956 are presented since the latest information will be given in detail elsewhere

Considerable advance has been made in the determination of the heat stability of Type A, strain 119 virus during the last 5 years at the Plum Island Animal Disease Laboratory In experiments designed to prepare, by heat

TABLE 4
EFFECTS OF HEAT ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS IN VESICULAR FLUIDS

Reference number	Virus history	Medium	Temperature and time	Route of inoculation and test animal	Results (infectivity)
22, 23	Bovine	None	31° C, 24 hours	Not mentioned, cattle and swine	Inactive
			37° C, 12 hours	Not mentioned, cattle and swine	Inactive
			37° C, 24 hours	Not mentioned, cattle and swine	Inactive
			60° C, ½ hour	Not mentioned, cattle and swine	Not completely destroyed
			70° C, ½ hour	Not mentioned cattle and swine	Inactive
16	Bovine	Not mentioned	45° C, 20 min 50° C, 15–30 min 60° C, 5–30 min 70° C, 10 min	Not mentioned Not mentioned Not mentioned Not mentioned	Inactive Inactive Inactive Inactive
17	Guinea pig	1 50, 0 85% saline	55° C, 15–40 min	Not mentioned	Inactive
		Phosphate solution	55° C, 2 min	Not mentioned	Inactive
15	Guinea pig	1 50, 0 85% saline	55° C, 20–45 min	Not mentioned	Not completely destroyed
		1 10, phosphate buffer, *p*H 7 6	55° C, 1 min	Not mentioned	Inactive
13	Guinea pig	1 100 phosphate buffer, *p*H 7 6	18–20° C, 25 days	Intradermal, guinea pig	Active
			32–34° C, 9 days	Intradermal, guinea pig	Active
			37° C, 3 days	Intradermal, guinea pig	Active
			37° C, 5 days	Intradermal, guinea pig	Inactive
11	Swine	None	37° C, 12 24 hours	Not mentioned	Inactive
11	Unidentified	Not mentioned	80–100° C, momentarily	Not mentioned	Inactive
			50–60° C, 3–9 days	Not mentioned	Inactive
12	Unidentified	Not mentioned	60–70° C, 5–15 min	Not mentioned	Inactive
13	Unidentified	Not mentioned	18–20° C, 14 days	Not mentioned	Active
21	Unidentified	None	53 and 70° C, 3, 6, and 9 days	Intravenously and scarification of oral mucosae, cattle	Inactive
17	Unidentified	Saline	55° C, 15–40 min	Not mentioned	Inactive
19	Unidentified	Not mentioned	30° C, 9 days 37° C, 12–24 hours	Not mentioned Not mentioned	Inactive Inactive

noninfectious antigen for use in the complement fixation test, infected tissue suspensions prepared from bovine tongue epithelium were heated at various temperatures for varying lengths of time. The infectivity and antigenicity of the tissue suspensions were tested subsequently. Although such attempts proved unsuccessful, in the preparation of a heat inactivated antigen the thermal death point of the virus in tissue suspensions was established.[27]

TABLE 5
EFFECTS OF HEAT ON THE SURVIVAL OF FOOT AND MOUTH DISEASE VIRUS IN DESICCATED MATERIALS

Reference number	Virus history	Medium	Temperature and time	Route of inoculation and test animal	Results (infectivity)
12	Guinea pig vesicular fluid	Not mentioned	70° C 2½ hours	Not mentioned	Active
			110° C 5 min	Not mentioned	Active
			120° C 3 min	Not mentioned	Active
			122° C 3 min	Not mentioned	Active
			130° C 1 min	Not mentioned	Active
20	Guinea pig vesicular fluid Type O	1:1000 in milk	90–95° C 5 min	Intradermal guinea pig	Active
			100° C 2 min	Intradermal guinea pig	Active
		1:100 in broth	100–110° C 5 min	Intradermal guinea pig	Active
			120° C, 3 min	Intradermal, guinea pig	Active
			130° C, 1 min	Intradermal guinea pig	Active
24	Guinea pig vesicular fluid, Type A	None	85–140° C 3–11 min	Not mentioned	Variable
			70° C 2½ hours	Not mentioned	Active
			122° C 3 min	Not mentioned	Active
17	Guinea pig vesicular fluid	1:50 dried at 37° C	18° C 2 112 days	Not mentioned	Active
			37° C 7 days	Not mentioned	Active
		Dried at 18° C	18° C, 3–6 months	Not mentioned	Active

Numerous methods of heating the infected tissue suspensions were used. One procedure that proved to be the most satisfactory and afforded critical control of temperature and constant agitation of both the contents of the waterbath and the container holding the virus suspension finally was adopted for all experiments.[28]

All infected tissue suspensions were prepared by grinding with Alundum in tryptose phosphate broth at pH 7.4 to a 10^{-1} dilution and by centrifuging. In the preliminary trials the supernatant fluids containing the virus were heated at 56° to 65° C for 30 min. Serial tenfold dilutions were prepared in a similar medium as described above, and inoculations were made in steers, suckling mice, guinea pigs, and tissue cultures of calf kidney epithelial cells. Evidence

of infectivity occurred in one or more of the test media used. In many instances more infected sites on steer tongue and larger numbers of tissue cultures showing cytopathogenic effects were observed after inoculations of the higher dilutions of virus than after inoculations of the lower dilutions. *Although this* reversal of infectivity was not always observed, it suggested an interference

TABLE 6
EFFECTS OF HEAT ON THE SURVIVAL OF FOOT AND MOUTH DISEASE VIRUS IN UNIDENTIFIED SOURCES OF VIRUS

Reference number	Medium	Temperature and time	Route of inoculation and test animals	Results (infectivity)
11	Not mentioned	45° C, 20 min 50° C, 15–30 min 60° C, 5–15 min 50° C, 2 days	Not mentioned Not mentioned Not mentioned Not mentioned	Inactive Inactive Inactive Active
25, 26	Not mentioned	56° C, 1/6–1 hour 60° C, 1/2 hour	Not mentioned	Retains most of initial complement fixing activity. Infectivity not completely destroyed
13	Not mentioned	37° C, 24–48 hours	Not mentioned	Inactive
17	Not mentioned Alkaline	31° C, 24 hours 37° C, 4–5 days	Not mentioned Not mentioned	Inactive Active
19	Not mentioned 1:500 in phosphate buffer, pH 7.6 Glycerol, pH 7.6 Glycerol in phosphate buffer, pH 7.6	37° C, few days 50° C, 15 min 37° C, 5 days 37° C, 24 hours 37° C, 24 hours	Not mentioned Not mentioned Not mentioned Not mentioned Not mentioned	Inactive Inactive Active Inactive Inactive

TABLE 7
EFFECTS OF HEAT ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS IN TISSUE EXTRACTS

Reference number	*Virus history*	*Medium*	*Temperature and time*	*Route of inoculation and test animal*	*Results (infectivity)*
13	Guinea pig vesicle coverings	Phosphate buffer, pH 7.5	18–20° C, 9–12 days	Not mentioned	Active
	Bovine tongue epithelium	Dextrose, sheep blood aerobically	37° C, 6 days	Intradermal, guinea pig	Active
			32–34° C, 10 days	Intradermal guinea pig	Active
			18–20° C,	Intradermal, guinea pig	Active

phenomenon such as has been reported in the literature with mixtures of active and inactive virus[29]

The studies devised for the determination of the thermal stability of the virus were divided into four groups of experiments, namely, the determination of infectivity of tissue suspensions after heating, using small and large inocula and laboratory safety tests to assure that cross infections from contaminated areas or infected animals did not provide a source of active virus to infect animals inoculated with heated suspensions, and complement fixation tests to determine the antigenic activity of the virus after heating

Small Inocula

After heating under the prescribed conditions of the experiments, the tissue suspensions were chilled to 4° C All unheated control preparations also were held at this temperature before both materials were diluted from 10^{-1} through 10^{-4} for infectivity tests

Adult steers were inoculated with 0 1 ml amounts of the suspensions by the intradermolingual route in 5 sites for each dilution, using 4 dilutions per tongue and 2 animals per sample Daily examinations for elevation in body temperature and vesiculation were made for as long as 14 days Animals not responding to primary inoculations of heated materials were challenged for susceptibility with active bovine adapted virus Serum samples also were collected during the course of the experiments and tested for the presence of complement fixing and virus neutralizing antibodies

Intradermal inoculations of the metatarsal pads of guinea pigs were made using 0 1 ml amounts of the inoculum and 10 animals per dilution Those guinea pigs not responding to the primary inoculations in the 14-day observation period were challenged with active guinea pig adapted virus

Suckling mice, 7 to 9 days of age, were also used as a test medium Each mouse was inoculated by the intraperitoneal route with 0 05 ml amounts of the materials using 10 mice per dilution The mice were observed daily for paralysis and death and final calculations were made at 14 days

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made in 48 hours

Representative results of infectivity in heated bovine tongue epithelial suspensions containing foot and mouth disease virus are given in TABLES 8, 9, and 10

In all instances virus suspensions heated at 56° C from 30 min through 24 hours were shown to be infectious for the bovine species and tissue culture The suckling mouse and guinea pig did not show evidence of infection in any of the experiments using heated materials and are not included in the results presented in TABLE 8

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of infectivity occurred in one or more of the test media used. In many instances more infected sites on steer tongue and larger numbers of tissue cultures showing cytopathogenic effects were observed after inoculations of the higher dilutions of virus than after inoculations of the lower dilutions. Although this reversal of infectivity was not always observed, it suggested an interference

Table 6
Effects of Heat on the Survival of Foot-and-Mouth Disease Virus in Unidentified Sources of Virus

Reference number	Medium	Temperature and time	Route of inoculation and test animals	Results (infectivity)
11	Not mentioned	45° C, 20 min 50° C, 15–30 min *60° C, 5–15 min* 50° C, 2 days	Not mentioned Not mentioned *Not mentioned* Not mentioned	Inactive Inactive *Inactive* Active
25 26	Not mentioned	56° C, ½–1 hour 60° C, ½ hour	Not mentioned	Retains most of initial complement fixing activity. Infectivity not completely destroyed
13	Not mentioned	37° C, 24–48 hours	Not mentioned	Inactive
17	Not mentioned Alkaline	31° C, 24 hours 37° C, 4.5 days	Not mentioned Not mentioned	Inactive Active
19	Not mentioned 1:500 in phosphate buffer pH 7.6 Glycerol pH 7.6 Glycerol in phosphate buffer pH 7.6	37° C, few days 50° C, 15 min 37° C, 5 days 37° C, 24 hours 37° C, 24 hours	Not mentioned Not mentioned Not mentioned Not mentioned Not mentioned	Inactive Inactive Active Inactive Inactive

Table 7
Effects of Heat on the Survival of Foot-and-Mouth Disease Virus in Tissue Extracts

Reference number	Virus history	Medium	Temperature and time	Route of inoculation and test animal	Results (infectivity)
13	*Guinea pig vesicle coverings*	Phosphate buffer, pH 7.5	18–20° C, 9–12 days	Not mentioned	Active
	Bovine tongue epithelium	Dextrose sheep blood aerobically	37° C, 6 days 32–34° C, 10 days 18–20° C, 25 days	Intradermal guinea pig Intradermal guinea pig Intradermal, guinea pig	Active Active Active

phenomenon such as has been reported in the literature with mixtures of active and inactive virus.[29]

The studies devised for the determination of the thermal stability of the virus were divided into four groups of experiments namely the determination of infectivity of tissue suspensions after heating using small and large inocula and laboratory safety tests to assure that cross infections from contaminated areas or infected animals did not provide a source of active virus to infect animals inoculated with heated suspensions and complement fixation tests to determine the antigenic activity of the virus after heating.

Small Inocula

After heating under the prescribed conditions of the experiments the tissue suspensions were chilled to 4° C. All unheated control preparations also were held at this temperature before both materials were diluted from 10^{-1} through 10^{-4} for infectivity tests.

Adult steers were inoculated with 0.1 ml amounts of the suspensions by the intradermolingual route in 5 sites for each dilution using 4 dilutions per tongue and 2 animals per sample. Daily examinations for elevation in body temperature and vesiculation were made for as long as 14 days. Animals not responding to primary inoculations of heated materials were challenged for susceptibility with active bovine adapted virus. Serum samples also were collected during the course of the experiments and tested for the presence of complement fixing and virus neutralizing antibodies.

Intradermal inoculations of the metatarsal pads of guinea pigs were made using 0.1 ml amounts of the inoculum and 10 animals per dilution. Those guinea pigs not responding to the primary inoculations in the 14-day observation period were challenged with active guinea pig adapted virus.

Suckling mice 7 to 9 days of age were also used as a test medium. Each mouse was inoculated by the intraperitoneal route with 0.05 ml amounts of the material using 10 mice per dilution. The mice were observed daily for paralysis and death and final calculations were made at 14 days.

In order to test the ability of as many media as possible to detect small amounts of active virus in suspensions that were subjected to heating an additional medium was included in these experiments. Bovine kidney epithelial cells were inoculated with 0.4 ml of the diluted materials. Final readings were made in 48 hours.

Representative results of infectivity in heated bovine tongue epithelial suspensions containing foot and mouth disease virus are given in TABLES 8, 9 and 10.

In all instances virus suspensions heated at 56° C from 30 min through 24 hours were shown to be infectious for the bovine species and tissue culture. The suckling mouse and guinea pig did not show evidence of infection in any of the experiments using heated materials and are not included in the results presented in TABLE 8.

All samples heated at 70° C for as long as 6 hours proved to be infectious for the steer whereas tissue cultures had the ability to detect active virus in suspensions that were heated no longer than 30 min. The mouse and guinea pig were refractory to infection with these materials (TABLE 9).

Several experiments were designed to remove extraneous tissue components by centrifugation and filtration and subsequent purification of the virus. When the virus was heated at 70° C for as long as 6 hours, it still proved to be infective for steers, and an interference phenomenon was also observed.

In one experiment a tissue suspension that had been treated at 56° C for 90

TABLE 8

EFFECTS OF HEATING AT 56° C FOR VARYING PERIODS OF TIME ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS* IN BOVINE TONGUE EPITHELIAL SUSPENSIONS, USING SMALL VOLUMES OF INOCULA

Duration of heat exposure	Dilutions of heated virus suspensions inoculated	Bovine tongue† (data on 2 steers)		Tissue culture†
30 min	10^{-1}	0/5	0/5	0/10
	10^{-2}	0/5	0/5	0/10
	10^{-3}	2/5	0/5	1/10
	10^{-4}	3/5	1/5	3/10
45 min	10^{-1}	5/5	0/5	0/10
	10^{-2}	5/5	3/5	3/10
	10^{-3}	5/5	0/5	2/10
	10^{-4}	2/5	0/5	6/10
1 hour	10^{-1}	1/5	0/5	0/10
	10^{-2}	0/5	1/5	0/10
	10^{-3}	2/5	3/5	0/10
	10^{-4}	3/5	0/5	2/10
2 hours	10^{-1}	0/5	0/5	1/10
	10^{-2}	0/5	2/5	4/10
	10^{-3}	1/5	0/5	2/10
	10^{-4}	0/5	0/5	5/10
4 hours	10^{-1}	2/5	2/5	0/10
	10^{-2}	0/5	1/5	3/10
	10^{-3}	0/5	0/5	6/10
	10^{-4}	0/5	0/5	3/10
6 hours	10^{-1}	3/5	2/5	0/10
	10^{-2}	2/5	0/5	3/10
	10^{-3}	1/5	0/5	2/10
	10^{-4}	0/5	0/5	0/10
12 hours	10^{-1}	2/5	5/5	2/10
	10^{-2}	0/5	5/5	2/10
	10^{-3}	0/5	1/5	4/10
	10^{-4}	0/5	0/5	0/10
18 hours	10^{-1}	1/5	2/5	0/10
	10^{-2}	2/5	0/5	1/10
	10^{-3}	0/5	0/5	3/10
	10^{-4}	0/5	0/5	0/10
24 hours	10^{-1}	3/5	1/5	0/10
	10^{-2}	4/5	0/5	0/10
	10^{-3}	1/5	0/5	1/10
	10^{-4}	4/5	1/5	0/10

* Control ID_{50} titers ranged between 6.9 and 8.8 for the bovine tongue and 6.9 to 7.2 for tissue cultures, as determined by the 50 per cent end point method of Reed and Muench.

† Number of sites or cultures infected/number of sites or cultures inoculated.

min and that had shown an interference phenomenon in cattle was stored as a 10^{-1} dilution at $-40°$ C for 6 months. When it was thawed and inoculated as before, similar results occurred.

The *pH* of the tissue suspensions after heating did not change significantly.

Reversal of virus infectivity was observed in approximately 30 per cent of these studies. In many experiments the 10^{-1} and 10^{-2} dilutions were devoid of demonstrable infectivity, whereas the 10^{-3} and 10^{-4} dilutions produced visible infection at times. This reversal was shown to diminish in degree as the temperature was increased.

TABLE 9

EFFECTS OF HEATING AT 70° C FOR VARYING PERIODS OF TIME ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS* IN BOVINE TONGUE EPITHELIAL SUSPENSIONS USING SMALL VOLUMES OF INOCULA

Duration of heat exposure	Dilutions of heated virus suspensions inoculated	Bovine tongue† data on two steers		Tissue culture†
30 min	10^{-1}	2/5		0/10
	10^{-2}	0/5	GI	2/10
	10^{-3}	0/5		2/10
	10^{-4}	4/5		4/10
1 hour	10^{-1}	2/5		0/10
	10^{-2}	0/5	—	0/10
	10^{-3}	0/5		0/10
	10^{-4}	0/5		0/10
1½ hour	10^{-1}	3/5	2/5	0/10
	10^{-2}	0/5	1/5	0/10
	10^{-3}	0/5	0/5	0/10
	10^{-4}	0/5	0/5	0/10
2 hours	10^{-1}	5/5	0/5	0/10
	10^{-2}	5/5	2/5	0/10
	10^{-3}	4/5	4/5	0/10
	10^{-4}	1/5	2/5	0/10
4 hours	10^{-1}	5/5	1/5	0/10
	10^{-2}	5/5	0/5	0/10
	10^{-3}	1/5	0/5	0/10
	10^{-4}	2/5	0/5	0/10
6 hours	10^{-1}	2/5		0/10
	10^{-2}	0/5		0/10
	10^{-3}	0/5	GI	0/10
	10^{-4}	0/5		0/10

* Control ID_{50} titers ranged between 7.5 and 8.5 for the bovine tongue and 6.4 to 7.1 for tissue cultures, as determined by the 50 per cent end point method of Reed and Muench.

† Number of sites or cultures infected/number of sites or cultures inoculated.

Key: GI, generalized lesions on tongue and feet.

Further attempts to inactivate the virus were made by increasing the temperature to 80° C, with exposure times of 30 min through 6 hours. Bovine tissue suspensions treated in this manner were infectious, but only for cattle and with material heated at 80° C for as long as 4 hours. The samples heated for 6 hours did not produce signs of the disease (TABLE 10).

Based on the above results obtained from experiments using small inocula, virus infectivity apparently was destroyed by heating at 80° C for 6 hours

Large Inocula

Additional materials were prepared and heated at 80° C for 6 hours When these were inoculated into steers by the subcutaneous, intramuscular, and intravenous routes in 50-ml amounts and by the intradermolingual route in 5 ml amounts, using 2 animals per route in each experiment, infectivity still was demonstrated Only cattle inoculated by the intravenous route proved to be refractory

TABLE 10

EFFECTS OF HEATING AT 80° C FOR VARYING PERIODS OF TIME ON THE SURVIVAL OF FOOT AND MOUTH DISEASE VIRUS* IN BOVINE TONGUE EPITHELIAL SUSPENSIONS USING SMALL VOLUMES OF INOCULA

Duration of heat exposure	Dilutions of heated virus suspensions inoculated	Bovine tongue† (data on 2 steers)	
30 min	10^{-1} 10^{-2} 10^{-3} 10^{-4}	0/5 2/5 0/5 0/5	2/5 5/5 3/5 0/5
2 hours	10^{-1} 10^{-2} 10^{-3} 10^{-4}	GL	GL
4 hours	10^{-1} 10^{-2} 10^{-3} 10^{-4}	GL	GL
6 hours	10^{-1} 10^{-2} 10^{-3} 10^{-4}	0/5 0/5 0/5 0/5	0/5 0/5 0/5 0/5

Experiments were then designed to heat the tissue suspensions at 85° C for
... as before Infectivity was
hours The suspen
tions for the bovine
... ments are presented in TABLE 11
virus suspensions heated
... ents were devised to test
this possibility The materials were diluted from 10^{-1} through 10^{-6} and using 2 steers per dilution, large doses of the heated suspensions were inoculated as previously described *In no case did any of the cattle develop the disease or* specific antibodies under the test conditions (TABLE 12)

It must therefore be assumed on the basis of these experiments that virus

contained in suspensions of infected bovine tongue epithelium did not survive heat treatment at 85° C for 6 hours.

Laboratory Safety Tests

Due to the extreme differences in the findings reported[7] and those appearing

TABLE 11

EFFECTS OF HEAT ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS IN BOVINE TONGUE EPITHELIAL SUSPENSIONS USING LARGE INOCULA IN CATTLE

Control ID50 titer of virus suspensions	Treatment temperature	Duration of heat exposure	Volume of 10 inoculum, ml	Route of inoculation	Results in 2 steers	
8.4	80° C	2 hours	50	IV	NVL	NVL
			50	SC	NVL	NVL
			50	IM	NVL	NVL
			5	IDL	GL	GL
8.1	80° C	6 hours	50	IV	NVL	NVL
			50	SC	GL	GL
			50	IM	NVL	NVL
			5	IDL	NVL	NVL
8.4	80° C	6 hours	50	IV	NVL	NVL
			50	SC	NVL	NVL
			50	IM	GL	NVL
			5	IDL	GL	GL
9.6	85° C	4 hours	50	IV	NVL	NVL
			50	SC	NVL	NVL
			50	IM	GL	GL
			5	IDL	NVL	NVL
8.1	85° C	4 hours	50	IV	NVL	NVL
			50	SC	NVL	NVL
			50	IM	NVL	NVL
			5	IDL	NVL	NVL
[illegible].7	85° C	6 hours	50	IV	NVL	NVL
			50	SC	NVL	NVL
			50	IM	NVL	NVL
			5	IDL	NVL	NVL
8.4	85° C	6 hours	50	IV	NVL	NVL
			50	SC	NVL	NVL
			50	IM	NVL	NVL
			5	IDL	NVL	NVL

Key: IV, intravenous; SC, subcutaneous; IM, intramuscular; IDL, intradermolingual; NVL, no visible lesions; GL, generalized lesions on tongue and feet.

in the early literature, it was necessary to determine that virus was not transferred accidentally to the animals given heat treated virus.

Routine laboratory operating procedures were followed as in all preceding trials. Animal quarters were decontaminated and stocked with steers for a 2 week observation period prior to inoculation of heated materials. The animals were observed daily for signs of disease and to test for residual active virus in the animal room. At the end of the 2 week holding period 1 of steers in each room was inoculated by the intradermolingual route, using

amounts of a 10^{-1} suspension of normal bovine tongue epithelium heated at 85° C for 6 hours. The remaining steer in the room served as a contact control. Other animals were then inoculated with suspensions of infected tongue epithelium heated at 85° C for 6 hours and known to be noninfectious on the basis of past trials. A 10^{-1} dilution was inoculated into each of 2 steers per route using 50-ml amounts by the intravenous, subcutaneous, and intramuscular routes and 5 ml by the intradermolingual route. Additional steers were then inoculated in a similar manner as described above with materials heated at 80° C for 6 hours and thought to be infectious as determined from previous experiments.

The animals were in suites composed of 2 rooms separated by a shower and airtight doors. Steers inoculated with a heated suspension of normal noninfected bovine tongue epithelium were located in one room while steers inoculated with heated infected suspensions were in the other. All steers were held for 2 weeks under these conditions. At the end of this period all animals inoculated with heated suspensions and not showing evidence of disease were challenged and held for an additional 2 weeks to determine possible spread of

TABLE 12

EFFECTS OF HEAT ON THE SURVIVAL OF FOOT-AND-MOUTH DISEASE VIRUS IN BOVINE TONGUE EPITHELIAL SUSPENSIONS USING LARGE INOCULA IN SERIAL DILUTIONS IN CATTLE

Control IDs title of virus suspension	Treatment temperature	Duration of heat exposure	Volume of inoculum (ml.)	Dilutions of heated virus	Route of inoculation	Results in each of 2 steers per dilution
8 1	85° C	6 hours	50 5	10^{-1} to 10^{-6} 10^{-1} to 10^{-4}	IM IDL	NVL NVL

Key: IM, intramuscular; IDL, intradermolingual; NVL, no visible lesions.

active virus to control animals inoculated with normal heated samples in the adjoining room of each suite.

Evidence of the control of the transfer of virus in laboratory animal rooms ... as well as by ... Cattle ... comple- ... hallenge ... eveloped

The only cattle to show neutralizing antibodies were ... the disease after inoculation with virus suspensions heated at 80° C for 6 hours. From these results it was concluded that when signs of the disease appeared in the inoculated animals or when sera from such animals were found to contain specific antibodies, the infection probably was due to viable virus contained in the inoculum, since the same operating procedures were used throughout the test. In addition, no steer inoculated with virus suspensions heated at 85° C for 6 hours showed signs of foot and mouth disease.

Complement Fixation Tests

Representative results of tests on ... complemen ... vity of tissue suspensions containing foot ... th

Complement fixing activity of the virus was not affected when the litter was heated at 56° C for as long as 24 hours, whereas heating at 70° C for 30 min diminished antigenicity significantly. All activity was destroyed when the virus suspensions were treated at 70° C for 2 or more hours.

Recently attention was given to the fact that heating one type of poliovirus would uncover antigens common to the other types.[36] Similar complement fixation tests were made and in no instance was crossing encountered with Types O and C hyperimmune serums and the Type A virus antigen being employed.

Suspensions were prepared from tongue epithelium harvested from cattle that had been inoculated with heated virus materials and that had shown le-

TABLE 13

COMPLEMENT FIXING ACTIVITY OF BOVINE TONGUE EPITHELIAL SUSPENSIONS CONTAINING FOOT AND MOUTH DISEASE VIRUS HEATED FOR VARYING LENGTHS OF TIME

Temperature and duration of heat exposure	Complement fixing activity* Antigen dilutions (1/… block)						Antigen controls
	Undiluted	1/2	1/4	1/8	1/16	1/32	
Unheated control	4	4	4	2	–	–	–
56° C 24 hours	4	4	4	3	–	–	–
Unheated control	4	4	4	2	–	–	–
70° C 30 min	3	–	–	–	–	–	–
70° C 2 hours	–	–	–	–	–	–	–
Unheated control	4	4	2	–	–	–	–
80° C 30 min		–	–	–	–	–	–
Unheated control	4	4	4	2	–	–	–
85° C 4 hours	–	–	–	–	–	–	–

* Against hyperimmune guinea pig serum.

Key: 4, no hemolysis (positive); 3 and 2, increasing degrees of hemolysis; –, complete hemolysis (negative).

sion. In these cases also no cross reactions with Types O and C hyperimmune serums were observed.

Discussion

Several important facts concerning the destruction of the infectious property of foot and mouth disease virus have been elucidated. In the tests reported in the early literature undiluted heated virus materials were inoculated in the test animals. In the studies herein reported[27] infectivity often was detected in animals inoculated with virus dilutions, whereas it was not detected in those given undiluted materials. This phenomenon was more in evidence when virus suspensions were heated at the lower temperatures.

The volume and route of inoculation were shown to be of extreme importance in the determination of the thermal death point of foot and mouth disease virus. Although evidence was presented at first for the absence of infection in cattle inoculated with small volumes of suspensions treated at 80° C for 6 hours, further and definite confirmation of virus destruction was needed. It

amounts of a 10^{-1} suspension of normal bovine tongue epithelium heated at 85° C for 6 hours. The remaining steer in the room served as a contact control. Other animals were then inoculated with suspensions of infected tongue epithelium heated at 85° C for 6 hours and known to be noninfectious on the basis of past trials. A 10^{-1} dilution was inoculated into each of 2 steers per route using 50 ml amounts by the intravenous, subcutaneous and intramuscular routes and 5 ml by the intradermolingual route. Additional steers were then inoculated in a similar manner as described above with materials heated at 80° C for 6 hours and thought to be infectious as determined from previous experiments.

The animals were in suites composed of 2 rooms separated by a shower and airtight doors. Steers inoculated with a heated suspension of normal noninfected bovine tongue epithelium were located in one room while steers inoculated with heated infected suspensions were in the other. All steers were held for 2 weeks under these conditions. At the end of this period all animals inoculated with heated suspensions and not showing evidence of disease were challenged and held for an additional 2 weeks to determine possible spread of

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active virus to control animals inoculated with normal heated samples in the adjoining room of each suite.

Evidence of the control of the transfer of virus in laboratory animal rooms may be checked by the appearance of specific signs and lesions as well as by the appearance of complement fixing and virus neutralizing antibodies. Cattle inoculated with the heat inactivated virus suspensions produced no complement fixing antibodies during the 14-day observation period prior to challenge. The only cattle to show neutralizing antibodies were animals that developed the disease after inoculation with virus suspensions heated at 80° C for 6 hours. From these results it was concluded that when signs of the disease appeared in the inoculated animals or when sera from such animals were found to contain specific antibodies the infection probably was due to viable virus contained in the inoculum since the same operating procedures were used throughout the test. In addition no steer inoculated with virus suspensions heated at 85° C for 6 hours showed signs of foot and mouth disease.

Complement Fixation Tests

Representative results of tests on the complement fixing activity of heated tissue suspensions containing foot and mouth disease virus are shown in TABLE 13.

Complement fixing activity of the virus was not affected when the latter was heated at 56° C for as long as 24 hours, whereas heating at 70° C for 30 min diminished antigenicity significantly All activity was destroyed when the virus suspensions were treated at 70° C for 2 or more hours

Recently attention was given to the fact that heating one type of poliovirus would uncover antigens common to the other types[30] Similar complement fixation tests were made, and in no instance was crossing encountered with Types O and C hyperimmune serums and the Type A virus antigen being employed

Suspensions were prepared from tongue epithelium harvested from cattle that had been inoculated with heated virus materials and that had shown lesions In these cases, also no cross reactions with Types O and C hyperimmune serums were observed

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Temperature and duration of heat exposure	Complement fixing activity* Antigen dilutions (10 stock)						Antigen controls
	Undiluted	½	¼	⅛	1/16	1/32	
Unheated control	4	4	4	2	—	—	—
56° C, 24 hours	4	4	4	3	—	—	—
Unheated control	4	4	4	2	—	—	—
70° C 30 min	3	—	—	—	—	—	—
70° C 2 hours	—	—	—	—	—	—	—
Unheated control	4	4	2	—	—	—	—
80° C, 30 min	—	—	—	—	—	—	—
Unheated control	4	4	4	2	—	—	—
85° C 4 hours	—		—	—	—	—	—

* Against hyperimmune guinea pig serum

Key 4 no hemolysis (positive), 3 and 2 increasing degrees of hemolysis —, complete hemolysis (negative)

Discussion

Several important facts concerning the destruction of the infectious property of foot and mouth disease virus have been elucidated In the tests reported in the early literature undiluted heated virus materials were inoculated in the test animals In the studies herein reported[7] infectivity often was detected in animals inoculated with virus dilutions, whereas it was not detected in those given undiluted materials This phenomenon was more in evidence when virus suspensions were heated at the lower temperatures

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must be remembered that, when the absence of infectivity in the steers was noted, the animals were inoculated by the intradermolingual route in 20 sites using 0.1 ml. of the inoculum per site. When the volume of inoculum of the heated virus suspensions was increased and several additional routes of inoculation were used, virus infectivity still was encountered with material heated at 80° C. for 6 hours. Only steers inoculated with 50 ml. each by the subcutaneous and intramuscular routes and with 5 ml. by the intradermolingual route became infected. Cattle inoculated by the intravenous route did not show evidence of infection and were shown to be fully susceptible to challenge in every case. Similar studies made with materials heated at 85° C. for 4 and 6 hours showed that in no case could infectivity be demonstrated in suspensions heated for 6 hours, whereas virus was still active when heated for 4 hours.

Numerous problems have been introduced because of these studies. There are certain federal regulations[?] concerning the importation of animal products from areas of the world where foot and mouth disease is enzootic. The regula... s during ... cts, and ... onsider ... -examination.

The more recent publication[?] with added revisions and supplements stipulates that fresh, chilled, or frozen glands, organs, extracts, secretions, or gall and like materials, dried blood, blood meal, tankage, lungs, meat meal, wool waste and manure, albumin, intestines, bone marrow, cooked sausage, cured ...

heating momentarily to a temperature of 156° F. (68.9° C.) or higher ... length of time or exact temperature for treating these products are not defined. In cases where the active fraction of certain glands or tissues is destroyed if heated above 122° F., then adjusting the *pH* of the product to 3.8 or 13.7 is allowed.

The findings also suggest that experimental results obtained in heat inactivation studies of other viruses should be reconsidered, taking the following factors into account:

Test medium. Sensitivity of the test medium should be such as to detect minute quantities of active virus in treated samples. In addition, as many susceptible hosts as possible should be used to test for innocuousness of the heated materials.

Dilution of treated samples. Samples should be tested for infectivity after dilution to eliminate the possibility of reading false negatives due to the interference phenomenon that may occur.

Volume and route of inoculum. Negative results may be obtained when using small volumes of test material that may contain minute amounts of active virus. If the volume of the inoculum is increased and inoculated via various routes, more active virus may be introduced. The various methods of inoculation also increase the probability of introducing virus by the most sensitive route, thus permitting the virus to multiply and produce infection.

Titer of inoculum Suspensions containing virus of high titer may require longer periods of exposure at higher temperatures than suspensions containing virus with a lower titer

Individual animal susceptibility within one species may also affect results

Acknowledgments

I acknowledge the aid of the director and other members of the staff of the Plum Island Animal Disease Laboratory in supplying numerous reference materials

Addendum

Recently, Cottral *et al* [32] reported the survival of foot and mouth disease virus in cured and uncured meat Carcasses were tested for residual virus after storage at 4° C It was found that virus was present in rib-bone marrow at 14, 60, and 73 days, and in lymph nodes, blood, and muscle at 60 days Muscle samples taken after 73 days of storage did not show active virus when inoculated into cattle

The chemical changes that occurred during ripening inactivated the virus in muscle but did not appreciably affect virus in lymph nodes large blood clots, or bone marrow This was due to acid formation and subsequent lowering of *p*H, which occurred most rapidly in muscle

It was concluded that meat from animals infected with foot and mouth disease virus was not rendered free of virus by the usual procedures of ripening, boning, salting, and storage The study showed that the virus could survive in certain tissues customarily included in such meat

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CORRELATION OF IONIZING RADIATION EFFECTS WITH PHYSICAL CHANGES IN VIRUSES*

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It has been known for many years that viruses can be inactivated or rendered incapable of producing disease by the action of ionizing radiations. Recently insight into the physical mechanism of virus inactivation has been realized through studies on the effect of X rays on tobacco mosaic virus (TMV).

To a certain extent the interpretation of the action of ionizing radiations on viruses depends upon the target theory. The essential feature of this hypothesis is that a biological effect ensues if the minimum requisite number of ion clusters is produced as a result of irradiation within a radiosensitive target. Since the probability of such an event or group of events depends upon the number of ion clusters produced per unit volume of material (which is a function of dose), upon the distribution of the ion clusters within the medium (which relates to the kind of radiation), and upon some function of the dimensions of the target, it is obvious, provided that the appropriate parameters can be assigned correct values, that the dose-response relationship for a given radiation can be interpreted in terms of target dimensions. This theory has been developed in great detail by Lea.[1] Complications recognized by Lea arise because of the fact that ion clusters can produce highly reactive substances, presumably free radicals, in the aqueous solvent that are capable, in principle, of causing biological damage to the target. Zirkle[2] attempted to modify the target theory to take into account ionizations occurring in the solvent in the immediate vicinity of the target. In general, it can be said that the target theory, while obviously reasonable in principle, is in many respects unsatisfactory in detail. This fact beclouds somewhat the interpretation of the inactivation of viruses by ionizing radiation in terms of specific mechanisms. An effort will be made to point out which of the conclusions derived from examination of X-ray inactivation data depend upon the validity of the target theory and which are entirely independent of it.

Early studies on the inactivation of viruses by X rays and other ionizing radiations were carried out by Lea and his associates.[1] Specific data on TMV were published by Gowen and Price,[3] by Lea *et al.*[4] and, more recently, by Buzzell *et al.*[5] As is illustrated by the data shown in FIGURE 1 for the inactivation of TMV by X rays, the logarithm of the biological activity surviving is a linear function of dose or, stated in other words, the fractional survival is an inverse exponential function of dose. This result is consistent with the target theory for the specific case that the occurrence of a single ion cluster within the radiosensitive target is sufficient to produce inactivation of the virus and is necessary if the single hit target theory is to be used, but is insufficient to establish that the target theory really is applicable.

Nevertheless, one can draw one necessary conclusion from the type of result

* This paper is Publication No. 67 of the Department of Biophysics, University of Pittsburgh.

... at each virus particle contains only one center *capable of causing* infection This conclusion is independent of the validity of the *target theory,* but it is contingent upon the ...
virus, *for example,*
virus Left unansi...
substance of a virus particle is contained in one physical piece or in several nonidentical physical pieces, all of which are necessary

Another approach to this problem depends upon the validity of the target theory Lea[1] showed that there was approximate correspondence between target volumes calculated by his detailed theory from inactivation responses of many viruses to ionizing radiations and the volumes of the virus-charac-

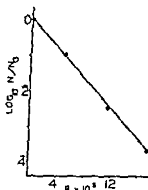


FIGURE 1 X irradiation of 2 per cent TMV in 25 per cent broth solutions Log10 of fraction remaining active is plotted against dose of irradiation in roentgens $\times 10^5$ Based on the data of Buzzell *et al*[5]

teristic particles Epstein[6] reconsidered the data available in the literature and showed that a better correlation exists between nucleic acid volumes of virus-characteristic particles and radiosensitive target volumes Buzzell *et al*[5] on the basis of more recent data on nucleic acid composition, virus size and radiation response and with a slightly different assumption concerning the number of ion pairs in a cluster, arrived at a precise correlation between target volume and nucleic acid volume for a number of viruses as shown in TABLE 1 This result, then means that essentially the entire nucleic acid content of the virus is the radiosensitive portion and is, therefore, the infectious substance of the virus-characteristic particle This conclusion however, is contingent upon the validity of the target theory and upon the correctness of the parameters chosen to calculate concentration of ion pair clusters from radiation dose

Pollard and Whitmore[7] bombarded oriented preparations of TMV nucleoprotein rods with 4 mev deuterons Their results are shown in FIGURE 2 Inactivation is at a maximum when the rods are oriented across the beam and at a minimum when they point toward it Since deuterons are densely ionizing particles, the considerations described in detail by Lea[1] lead to the conclusion

that the biological effect should be proportional to the cross section of the target presented to the deuteron beam Consequently, the results of Pollard and Whitmore are consistent with the view that the target cross section pre

TABLE 1
VIRUS TARGET VOLUMES (×10[illegible] ml)

Virus	Target vol.	Nucleic acid vol.	Total vol.
Tobacco mosaic	4 1	3 0	50
Tobacco necrosis	1 5	1 5	8 1
Tobacco ringspot	2 8	3 2	8 1
Tomato bushy stunt	2 9	2 7	15 6
T2 bacteriophage	52	55	150
T6 bacteriophage	52	55	150
T7 bacteriophage	17 5	18	47
Shope papilloma	3 9	4 2	49
PR8 influenza	18	13	294

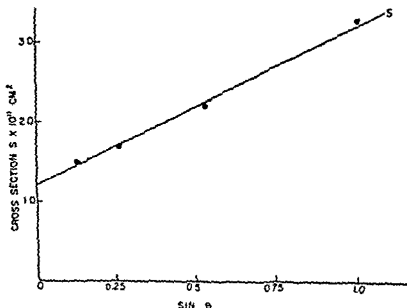


FIGURE 2 Plot of the effective sensitive area S against the sine of the angle of exposure of dry TMV preparations to deuterons

sented to the deuteron beam is at a maximum when the rodlike virus partic[les] are perpendicular to the beam and at a minimum when the rodlike particles point toward the beam From this, Pollard and Whitmore [...] that the radiosensitive target in a TMV particle is itself a rod conta[ining] the characteristic particle rod and lying parallel to it This [is com]pletely consistent with the suggestion made earlier by Epstein[4]

acid is the radiosensitive target, because Hart[8] and Schramm *et al.*[9] showed independently that the ribonucleic acid (RNA) of TMV is a cylinder lying somewhere near the center of the TMV particle and with the same axis.

Two lines of evidence converged in 1956 to establish firmly the fact that the radiosensitive target of TMV is the RNA. The first of these was the independent discovery made by Fraenkel-Conrat[10] and by Gierer and Schramm[11] that highly purified preparations of TMV RNA are infectious. The second, a description of which was in press at the time that these discoveries were first detailed in print, was the demonstration by Lauffer *et al.*[12] of physical damage to the RNA of TMV as a result of irradiation.

Two per cent solutions of TMV were irradiated with various doses of 0.2 Å X rays, and the nucleic acid was subsequently isolated by the method of heat denaturation described by Hopkins and Sinsheimer.[13] Freedom from protein was verified by noting that the ratio of the extinction coefficients at 2600 and 2800 Å agreed close [illegible] that of virus protei[illegible] from X irradiated [illegible] unirradiated virus.[12] The most probable interpretation of decrease in intrinsic

TABLE 2[12]

INTRINSIC VISCOSITY OF TOBACCO MOSAIC VIRUS NUCLEIC ACID FOLLOWING X IRRADIATION

Intrinsic viscosity (mL/gm.)	Radiation dose (10^6 r)	Intrinsic viscosity (mL/gm.)	Radiation dose (10^6 r)
84	0	65	0.46
77	0	37	1.85
74	0	40	1.85

viscosity is that the nucleic acid is depolymerized. This is true whether the nucleic acid exists in solution in the form of a slender rod or in the form of a randomly kinked chain. There is, of course, in principle, the possibility that decrease in viscosity could be brought about by converting a rigid structure into a less rigid one, presumably by breaking a cross bonding linkage. This differs only in detail from the kind of break that leads to depolymerization. There is the further possibility, in principle, that a decrease in intrinsic viscosity could be brought about by a decrease in hydration. However, such an inter[illegible] to be correct because of the fact that it would be [illegible]

the best interpretation of the decrease in intri[illegible] from irradiation of TMV is that the nucleic acid is depolymerized.

The breakage comes about, not by fracture of the TMV rods themselves, but by radiation damage done inside the intact virus particle. This is shown by the fact that heavily irradiated TMV possesses essentially the same sedi[illegible] and electron image size as unirradiated [illegible] hen the [illegible] has [illegible] is to

a similar decrease in the intrinsic viscosity of subsequently isolated RNA.[14] As pointed out by Lauffer *et al.*,[12] "An important deduction from these findings is that the biological activity of tobacco mosaic virus depends on the physical

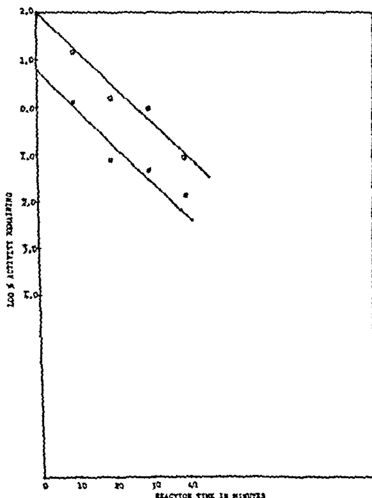


FIGURE 3. Inactivation of X rayed T5 bacteriophage in 0.8 per cent broth solution at 70° C.[22] Symbols: *circles*, controls; *squares*, sample of T5 bacteriophage previously irradiated in 0.8 per cent broth by 140 kv X rays for 4 hours.

intactness of the nucleic acid." This constituted the first demonstration known to me of a physical effect associated with inactivation of TMV by irradiation.

Buzzell and Lauffer[22] found that when T5 bacteriophage was inactivated by 140 kv X rays in 4 per cent broth solution, the data fell on a straight line whe the logarithm of activity remaining was plotted against dose. However, wh

the irradiation was carried out in 0.8 per cent broth solution, results were obtained that fell on a curved line, indicating a higher rate of inactivation at extreme X ray exposures. Survivors of X ray inactivation were subsequently

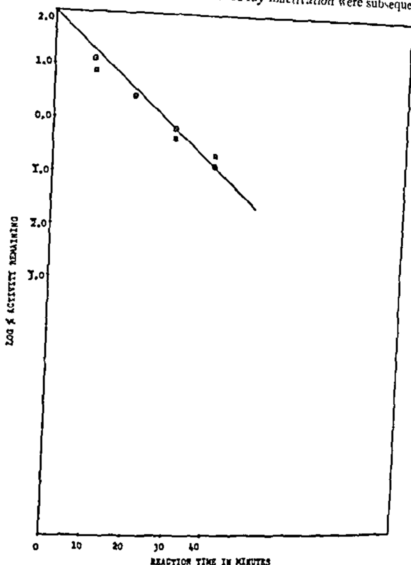


FIGURE 4. Inactivation of X rayed T5 bacteriophage in 0.8 per cent broth solution. Symbols: *circles*, controls; *squares*, sample of T5 bacteriophage previously irradiated in 0.8 per cent broth by 140 kv X rays for 6 hours.

inactivated by heating at 70° C. As shown in FIGURES 3 and 4, survivors from irradiation in 0.8 per cent broth solution showed greater sensitivity to heat than unirradiated virus, but survivors from irradiation in 4 per cent broth solution showed the same sensitivity to heat as unirradiated virus. These results were interpreted to indicate sublethal injury of those virus particles irradi-

ated in 0.8 per cent broth solution, injury presumably caused by indirect action of the radiation. Similar studies were carried out on TMV previously subjected to 5 times the average lethal dose of X irradiation.[8] In this case both the irradiation and the thermal stability measurements were carried out in 25 per cent broth solution. The data are shown in FIGURE 5. There is no detectable difference between the thermal stability of unirradiated virus and of survivors from irradiation. Since the target size in TMV is about one twelfth the size of the total virus particle, particles subjected to 5 times the average lethal dose should suffer an average of 55 nonlethal hits in the protein portion. Such hits apparently do not affect the virus rods' resistance to thermal inactivation, thereby demonstrating that the protein portion of the virus is far less important biologically than the nucleic acid. This result fits perfectly with the known ability of nucleic acid to produce infection in the absence of protein.

Further studies on the depolymerization of TMV by the action of X rays have been carried out by means of ultracentrifugation.[14,15] It was found that

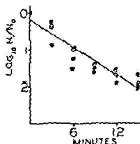


FIGURE 5. Inactivation of TMV at 80° C. in 25 per cent broth solution. Symbols: *open circles*, unirradiated virus; *solid circles*, survivors of 5 D_0 of X rays. Based on data of Buzzell *et al.*[8]

nucleic acid isolated by the phenol method from unirradiated TMV exhibited a reasonably homogeneous component in the ultracentrifgue with a sedimentation coefficient of 31 svedbergs, plus additional amounts of inhomogeneous material with lower sedimentation coefficients. The fraction of material in the s 31 component corresponded to the fraction of the TMV particles in the preparation having the standard 3000-Å length, and the amount of slower moving material corresponded to the amount of fragmented material in the original virus preparation. Schuster *et al.*[16] have also obtained RNA from TMV with a sedimentation coefficient essentially equal to that obtained in this study. They calculated from a combination of sedimentation and viscosity data that the molecular weight of this component is about 2 million, a value that corresponds to the total RNA content of a TMV particle.

When such virus preparations were irradiated, the amount of material in the s 31 component of the subsequently isolated RNA was found to decrease.[14,15] In FIGURE 6 the decrement is shown plotted on a logarithmic scale as a function of dose of radiation. These data are virtually superimposable upon the TMV inactivation data. When the virus was irradiated at dry ice temperature the rate of inactivation was essentially the same as that for TMV irradiated in solution, but the rate of breakage was considerably slower, as shown in FIGURE

6 A similar slower rate of RNA breakup was observed following irradiation of wet gels of TMV These data therefore indicate clearly that infectiousness is correlated with the amount of full length RNA This result has direct bearing upon the questions of whether the infectious unit of TMV is the total RNA content and whether this material is in one physical piece

Gierer[19] has demonstrated a correlation between the infectiousness of RNA and the amount of material in the undegraded form Further evidence from the effect of dilution on number of lesions supports this conclusion Cart

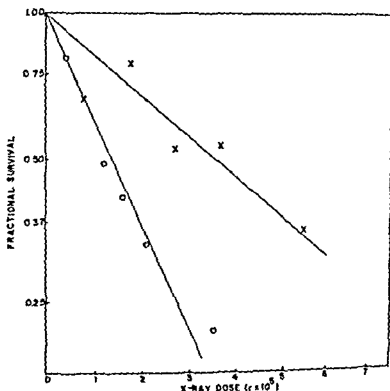


FIGURE 6 Fraction of originally intact RNA of TMV remaining unbroken after various dosages of X rays[14] Symbols *circles*, TMV irradiated *in solution* *crosses*, TMV irradiated in frozen state

has observed that the
of lesions pro-
plants is com

parable to the effect of dilution of the virus itself on lesion count In other words, the slope of the dilution curve for the infectious nucleic acid is the same as that for the virus *It has been shown by* Lauffer and Price[20] that the dilution curve *for many* viruses can be accounted for in terms of the theory that infection is caused by a single particle entering a susceptible cell the probability of an infection being *determined by the chance* occurrence of at least one infectious particle *in* the volume of material that comes into contact with a susceptible center

Parenthetically, the conclusion reached by Lauffer and Price that this simple dilution theory is to be preferred to available alternatives has been subjected to considerable criticism, particularly by Kleczkowski.[22] It is necessary to agree that mathematical analyses of dilution data cannot settle the question of the mechanism of infection of a tobacco plant by TMV. The reason is that an alternative assumption, the so-called Gaddum theory, based on the hypothesis that the logarithm of the susceptibility of the host to the virus is distributed according to a normal curve, can also account for the results. Lauffer and Price did not base their preference for the simple-dilution (Poisson) theory primarily on the shape of the dilution curve. They were more impressed by results obtained with mixed inocula of TMV and aucuba mosaic virus. These mixed inocula were applied at various concentrations to leaves of *Nicotiana langsdorfii* and the resulting lesions were analyzed by subinoculation to *N. sylvestris* plants for TMV, for aucuba mosaic virus, and for mixed infections.

TABLE 3
DISTRIBUTION OF MIXED INFECTIONS

Log. concentration gm./cc.	Distribution of virus recovered			Not infected	Total	Per cent mixtures				
								Expected		
									Gaddum theory	
	TMV alone	AMV alone	TMV AMV			Observed	Corrected*	Poisson theory	†	‡
−2.5	58	9	32	1	100	32	82	100	100	100
−4.5	58	27	12	3	100	12	44	53.9	100	100
−6.5	98	101	3	6	208	1.5	1.5	0.84	58.5	100

* Corrected on the assumption that numbers of TMV and AMV lesions should be the same at all concentrations and that, therefore, the excess number of TMV lesions at the higher concentrations represents initially mixed infections in which TMV crowded out AMV.

† Fifty per cent concentration assumed to be 10^{-4} gm./cc. with 10^{1} particles coming into contact with susceptible area.

‡ Fifty per cent concentration assumed to be 10^{-4} gm./cc. with 10^{4} particles coming into contact with susceptible area.

The expected fractions of mixed infections at various dilutions may be calculated from the opposing theories. The results are shown in TABLE 3. It must be pointed out that the parameters originally used in calculating the expected results from the Gaddum theory gave that hypothesis the benefit of every doubt. Schramm and Engler[73] have since shown that infection can be produced when as little as 5×10^{-18} grams of TMV are applied to susceptible plants. It can be estimated that the amount of virus applied in the experiments of Lauffer and Price[20] was about 10^{-19} gm./lesion at the 50 per cent dose. Therefore, on the basis of the Gaddum theory, the number of infectious particles entering a cell at the 50 per cent dose must have been at least 10^{4}. Based on this parameter, the expected fractions of mixed infections are shown in the right hand column of TABLE 3. Originally there was one hidden assumption in this interpretation, to the effect that both TMV and aucuba mosaic vi- can grow in the same cell. Bendix[23] showed by a microtechnique that m

infection can indeed arise from simultaneous introduction into a single cell of a mixed inoculum. In the light of these new findings it is obvious that application of the Gaddum theory to TMV is not valid. The simple-dilution theory accounts for all of the facts.

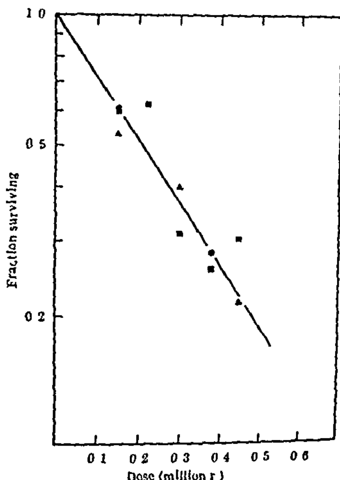


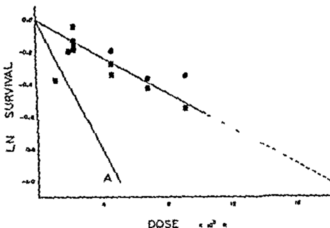
FIGURE 7. X ray inactivation of TMV. The curve represents a summary of 3 separate experiments (frozen or dried samples) as indicated by different symbols.[24] Reproduced by permission from *Nature*.

To resume since the dilution curve for the isolated nucleic acid parallels that of TMV itself, it follows if this theory is correct, that the infectiousness of the nucleic acid is contained in a single physical piece. If more than one piece of nucleic acid whether of the same or of different kinds were required to initiate infection, the slope of the dilution curve would be greatly different.

Ginoza and Norman[24] have shown that isolated TMV nucleic acid is inactivated by 50 kv X rays. Their results for both dry and frozen nucleic acid are shown in FIGURE 7. The minimum lethal dose is virtually the same as for inactivation of TMV itself. When inactivation was carried out in the presence

of 2 per cent glutathione, a twofold decrease in radiation sensitivity was observed.

Further study carried out in my laboratory[25] shows that isolated nucleic acid is also depolymerized when irradiated. In this case, however, there are complications. When RNA at a concentration of 0.04 mg./ml. was irradiated at dry ice temperature, D_0 for breakage was approximately the same as D_0 for breakage of RNA in irradiated and frozen TMV, namely, 5×10^5 r. When the concentration was increased to 0.2 mg./ml., or when O_2 was removed from the solution at a concentration of 0.04 mg./ml., the average lethal dose was raised to about 18×10^5 r, as shown in FIGURE 8. If, prior to freezing, oxygen was removed from the concentrated solution, D_0 was increased still further to approximately 34×10^5 r, as shown in FIGURE 9. However, D_0 for inactivation



remains essentially constant regardless of concentration or oxygen content. Preliminary values obtained in our laboratory place D_0 for inactivation of frozen RNA at approximately 1×10^5 r, a value somewhat less than that reported earlier by Ginoza and Norman[4] for both frozen and dried RNA. If RNA irradiated under conditions of maximum protection at dry ice temperature is subsequently heated for 2 min. at 40° C, the extent of breakage is much increased, as shown in FIGURE 9.

It is evident from all of the studies discussed that X rays inactivate TMV, that they depolymerize the nucleic acid when virus is irradiated, that they inactivate isolated nucleic acid, and that they also depolymerize isolated nucleic acid. However, the relationships between these various phenomena are not simple. As far as virus inactivation goes, D_0 seems to be essentially the same for virus irradiated in solution, virus irradiated when dry,[1,4] and virus irradiated when frozen. The D_0 for inactivation of nucleic acid seems to be within experimental uncertainty the same as that for the virus, whether the

nucleic acid is irradiated in the dry state or in the frozen state. Target volumes calculated by the theory of Lea[1] from the inactivation data agree reasonably well with the nucleic acid volume. All of these results tempt one to conclude that inactivation of either TMV or TMV nucleic acid results from the generation of a single ion cluster anywhere within the nucleic acid. Presumably this means that, if any chemical change occurs anywhere within the nucleic acid, the virus particle or the isolated RNA particle is rendered unable to induce infection. One is tempted to ascribe this action to the direct effect because D_0 is essentially the same whether the irradiated material is, dry, in solution, or frozen. However, it must be conceded that biological activity measurements are not sufficiently precise to rule out absolutely the possibility that D_0

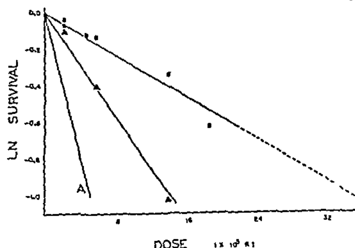


FIGURE 9. Natural logarithm of fraction of RNA from TMV remaining unbroken follow ... following heating for 2 min. at 40° C. ... and Lauffer, unpublished data.) Line ... 2 mg./ml. with O_2 removed (triangles ...

might be somewhat higher for irradiation in the dry state, thus allowing for some indirect action in the inactivation. ... molecated

Th...

liquid state ...

is irradiated in the frozen state, the D_0 for breakage is greater than the ... RNA de... killing. This latter result, taken by itself, is not very surprising. Since ... polymerization should follow only when a break occurs in the backbone, ... target, ... obviously much smaller than the total RNA target, ... dence be

... s

radiated in solution is perhaps the surprising ... suspect because of persistent decrease in the yield of extracted RNA with increasing X ray dose, as observed by Englander *et al.*[28] The results obtained with frozen RNA are complicated in still another respect. Breakage is con

centration-dependent and varies with the amount of oxygen present in the solution prior to freezing Furthermore it can be developed by subsequent heating These results suggest that indirect action might play a role in RNA breakage even though inactivation seems to be largely the result of direct action *They also might mean that direct action involves the formation of free radicals on the RNA, which leads to breakage only in the presence of* oxygen, as suggested by the theoretical considerations of Alexander[27] The latter view can also account for the inactivation in the presence of glutathione, as observed by Ginoza and Norman[24]

It seems to be perfectly clear that inactivation of TMV is the result of radiation action on the nucleic acid This action sometimes leads also to depolymerization of the nucleic acid However, the data indicate that the actual breaking of the chain is not necessary for inactivation

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THERMAL INACTIVATION OF ANIMAL VIRUSES

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The published reports on the thermal inactivation of animal viruses give a picture of a complex phenomenon However much of this may be the result of a lack of thoroughness and accuracy in experimentation rather than of inherent complexity in the system Nevertheless, there are several well-established characteristics of animal virus thermal inactivations that do indicate some complexity, at least Examples are (1) the Q_{10} for virus inactivations often varies greatly with temperature (2) first-order kinetics are obeyed for a few $\log_{10}$ units only, there being a relatively thermal resistant 10 per cent or less of most virus cultures and (3) at certain temperatures two virus functions (for example, infectivity and serologic properties) are lost at the same rate, while at other temperatures the two functions are lost at different rates

In recent years several thorough studies on the subject of animal virus thermal inactivations have appeared and there seems to be a common pattern to all of these In this paper I shall attempt to identify the general characteristics of animal virus thermal inactivations as elucidated by these studies and try to develop a simple unified hypothesis relating all or most of the facts Before turning to the details of virus inactivations it will be profitable to review what is known of the thermal inactivation of the component parts namely protein and nucleic acids

The Eyring theory of absolute reaction rates has proved to be very useful in elucidating the mechanism of many simple chemical reactions Furthermore it has been applied with some success to an understanding of the inactivation of enzymes and protein denaturation[1] Written in its usual form the Eyring equation[2] relating the reaction rate constant k to thermodynamic parameters is

$$k = \frac{kT}{h} e^{-\Delta H\ddagger/RT} e^{\Delta S\ddagger/R} \tag{1}$$

where k is Boltzmann's constant h is Planck's constant R is the gas constant, T is absolute temperature and $\Delta H\ddagger$ and $\Delta S\ddagger$ are, respectively, the heat and

breaking of a single chemical bond they are due to the breaking of many chemical bonds most probably hydrogen bond the breaking of which is associated[4] with about 5 kcal mole $\Delta H\ddagger$ and 12 cal mole degree $\Delta S\ddagger$ Stearn's hypothesis based upon hydrogen bond breakage accounts satisfactorily for most protein inactivation, however notice the values for ribonuclease (RNase) in TABLE 1

Turning next to desoxyribonucleic acid (DNA) inactivations, it is found that, like most protein inactivations, these are associated with high $\Delta H\ddagger$ and $\Delta S\ddagger$ values. Rice and Doty[3] have shown that DNA thermal denaturation has a $\Delta H\ddagger$ of 93 kcal./mole and a $\Delta S\ddagger$ of 220 cal./mole/degree. These authors give additional evidence that the actual mechanism involved is probably the breakage of hydrogen bonds followed by a collapse of the DNA structure. Zamen

TABLE 1

THERMODYNAMIC PARAMETERS CHARACTERIZING THE THERMAL INACTIVATION OF VARIOUS BIOLOGICAL MATERIALS

Heat Inactivation Constants

	$\Delta H\ddagger$ $\times 10^3$ cal./mole	$\Delta S\ddagger$ cal./mole/°C.	Reference No.
I. Proteins			
RNase	33	19	13
Lipase	45	68	1
Pepsin	56	113	1
Hemoglobin	76	153	1
Egg albumin	132	316	1
Peroxidase	185	466	1
Hemolysin, goat	198	537	1
II. Desoxyribonucleic acid			
DNA denaturation	93	220	3
III. Ribonucleic acid			
TMV RNA	19	−19	6
IV. Bacterial viruses			
T1	95	207	19
T2	72	139	19
T3	105	246	19
M1	76	165	20
M3	87	195	20
M4	136	347	20
Strep. phage—high temp.	76	165	12
Strep. phage—low temp.	11	−32	12
V. Plant viruses			
TMV prot. denat.	150	350	19
TMV high temp.	105	234	19
TMV low temp.	17	−21	19
TMV in formalin	23	3	21
TMV RNA	19	−19	6
SBMV	25	−5	19
SBMV large antigen	17	−22	19
SBMV small antigen	159	370	19
VI. Animal viruses			
FMDV—low temp.	26	7	8
FMDV—high temp.	113	280	8
Polio—slow component	30	—	9
Polio in formalin	30	—	10
Polio dry	29	21	25
Polio vaccine	50	—	27
Coxsackie	200	—	24
Measles—low temp.	18	—	26
Measles—high temp.	70	—	26
APC	50	—	23
Vaccinia	20–90	—	7
Influenza A—hemagg.	170	450	30
Influenza B—hemagg.	340	950	30
NDV—hemagg.	125	300	30
NDV infect.—high temp.	125	300	14
NDV infect.—low temp.	29	30	14

hof *et al*[4] have extended this type of study to the inactivation of transforming principle, and find that DNA thermal denaturation and thermal inactivation of transforming principle probably occur at the same rate More recently Lehrman and Tolmach[5] have completed a far more thorough study of the kinetics of transforming principle inactivations and confirm this result among other things

Unlike DNA and protein inactivations, ribonucleic acid (RNA) inactivation in the temperature range where it has been measured is not associated with high values of $\Delta H\ddagger$ Ginoza[6] finds for tobacco mosaic virus (TMV) RNA a value of about 20 kcal mole for $\Delta H\ddagger$ and −19 cal /mole/degree for $\Delta S\ddagger$ These values are incompatible with a hydrogen bond breakage mechanism and

also has produced independent evidence that RNA inactivation actually is associated with random breaking of the RNA chain (Ginoza, unpublished work)

In summary, then DNA and protein thermal inactivations seem to be alike in that they are associated with a high $\Delta H\ddagger$ and $\Delta S\ddagger$ and they occur primarily through breakage of hydrogen bonds resulting in a collapse or drastic refolding of the secondary structure of the molecule However breakdown of the molecule into subunits is not observed[3] On the other hand RNA inactivations have low $\Delta H\ddagger$ and negative $\Delta S\ddagger$ values and occur by a chemical reaction at a single site, which splits the molecule It does not seem probable that any secondary structure that RNA might possess at the time of inactivation would be seriously rearranged by this process

With this background the heat inactivation of animal viruses now can be examined In doing so the thorough studies of Kaplan on vaccinia virus,[7] of Bachrach and his co-workers on foot and mouth disease virus[8] and of Youngner[9] and Timm and his collegues[10] on polio virus will be utilized

Considering first the shape of thermal inactivation curves it is seen from FIGURE 1 (taken from Kaplan[7]) that vaccinia is inactivated in a two-component fashion each component following first-order kinetics This type of curve is representative of virus thermal inactivations in general Next it may be seen from the figure that the intercept resulting from extrapolating the slow inactivating component back to the ordinate is a function of the temperature at which the inactivation occurs Furthermore as the inactivation temperature is lowered this extrapolated intercept approaches 100 per cent This fact is far more evident from the data on polio- and foot and mouth disease viruses It follows from this fact that for temperatures lower than a certain value, inactivation curves have one component only that inactivates with first-order kinetics This is seen clearly in the data on foot and mouth disease virus referred to previously

Concerning the temperature dependence of these reactions it is seen (for example in FIGURE 2 taken from the data of Bachrach *et al*[8] on foot and mouth disease virus) that in the low temperature range where there is one component only to the inactivation curves, the $\Delta H\ddagger$ is constant and is about 20 to 30 kcal mole In the high temperature range where there are two components to

activation curves, the Δ... one shown in FIGURE 2) slow inactivating compc... temperature range (although there are not sufficient data bearing on this point to date) TABLE 2 summarizes these essential features of animal virus thermal inactivations

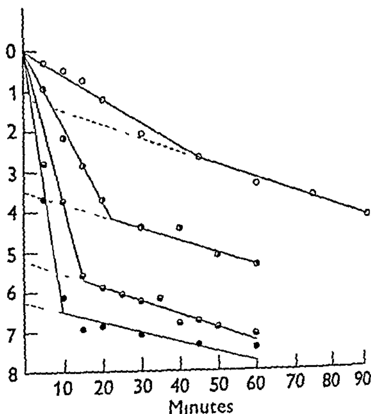


FIGURE 1 Thermal inactivation of vaccinia virus Reproduced by permission from the *Journal of General Microbiology*[7]

Since the above-mentioned features seem to be general for most, if not all, animal virus inactivations it is possible that they are merely different manifestations of some basic property of viruses, or even of biological type molecules in general In this connection, it might be mentioned that some, if not all of the characteristics summarized in TABLE 2 are seen for TMV thermal inactivations (two component curves, intercept varying with temperature, low ΔH‡ ... ΔH‡ at high temperatures according to Pollard

Before attempting to develop a hypothesis of animal virus thermal inactivations several simple explanations for these phenomena should be considered and it should be shown where these explanations fail to accord with the facts. Generally, two-component inactivation curves are explained on the basis of inherent population heterogeneity. However, this explanation cannot account for the fact that the extrapolated intercept of the second component is not independent of temperature.

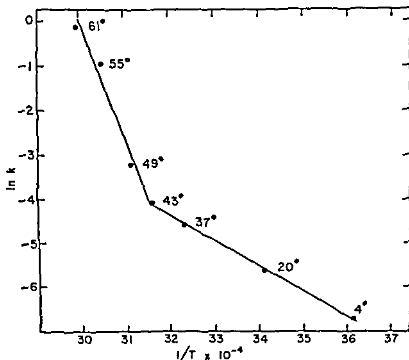


Figure 2. Temperature dependence of the thermal inactivation rate constant for foot and mouth disease virus. Reproduced by permission from *Proceedings of the Society for Experimental Biology and Medicine*.

TABLE 2
CHARACTERISTIC FEATURES OF ANIMAL VIRUS THERMAL INACTIVATIONS

	Low temperature	High temperature
Kinetics	1 component first order	2 components—each first order
ΔH‡	20 to 30 kcal/mole	20 to 30 kcal/mole slow component 50 to 100 kcal/mole fast component
Intercept of slow component	(no second component exists)	increases with decreasing temperature approaching 100 per cent

An apparent explanation for the fact that at low temperatures the $\Delta H\ddagger$ for the inactivation process is low, while at high temperatures the $\Delta H\ddagger$ is high is that in the different temperature ranges, different components of the virus are being inactivated An example of this would be inactivation by protein denaturation in the high temperature range and inactivation by nucleic acid destruction in the low temperature range There seems to be some actual evidence for this view in the fact, referred to by Youngner,[8] that only at high temperatures does poliovirus lose infectivity and serologic properties simultaneously Nevertheless, this parallel paths explanation does not predict and is not consistent with, the two-component inactivation curves encountered at higher temperatures

A model is presented that is believed to account for the aforementioned features characterizing animal virus inactivations The mathematical model is by no means unique, and the physical interpretation given it is far more specific than the mathematical model necessitates It is assumed that heat inactivation of animal viruses occur primarily through damage to the nucleic acid Consider the nucleic acid of the virus to exist in two viable forms that are inter

... the other form A Presumably

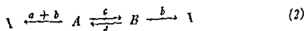
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rthermore there

are considered to be two mechanisms for the thermal inactivation of the virus nucleic acid One is by a route analogous to protein or DNA inactivation namely, by breaking of a large number of hydrogen bonds followed by collapse of the secondary structure Hence, this will be called the collapse mechanism The other inactivation mechanism is by means of some chemical reaction leading to a break or change in the nucleic acid chain at a single point similar to TMV RNA inactivations While the collapse mechanism would be charac terized

ondary

little eff



$$X \xleftarrow{a+b} A \underset{d}{\overset{c}{\rightleftharpoons}} B \xrightarrow{b} X \quad (2)$$

where A and B are the two viable forms of virus nucleic acid X represents in ... are the rate constants for the collapse mechanism and the

e

$$S = \frac{1}{d}\{e^{R_1 T}[C_1(a+b+c+d+R_1)] + e^{R_2 T}[C_2(a+b+c+d+R_2)]\} \quad (3)$$

where

$$S = A + B, \qquad R_{1,2} = \tfrac{1}{2}[-(a + 2b + c + d) \pm \sqrt{(a + c + d)^2 - 4ad}],$$

$$C_1 = \frac{1}{R_1 - R_2}[dB_0 - (a + b + c + R_2)A_0],$$

and $C_2 = A_0 - C_1$

Several special solutions of this equation are of interest. Since the rate constant b (that is, the constant for the chain break mechanism) has a lower $\Delta H\ddagger$ than does the constant a, there will be a low temperature range in which b is greater than a. For this case the equation simplifies to

$$\frac{S}{S_0} \cong e^{-bt} \tag{4}$$

which is exactly the equation required of the low temperature region. For other conditions two-component curves are expected and if, for the high temperature region in which a is greater than b, it is required also that d is negligible, a clear solution is obtained

$$S = e^{-(a+b+c)t}\left[\frac{aA_0}{a + c}\right] + e^{-bt}\left[B_0 + \frac{cA_0}{a + c}\right] \tag{5}$$

It is evident that not only are these curves two-component, but that the extrapolated intercept of the slow inactivating component with the ordinate can be a function of temperature of inactivation. Also, the rate of inactivation of the slow component is controlled by the same rate constant that controls inactivations in the low temperature range. Therefore, a unified hypothesis based on the assumption that virus nucleic acid exists in two viable states can account for the observed facts.

There are several published reports on animal virus thermal inactivation that, at the time seemed puzzling but, viewed in the light of the above hypothesis, seem simpler. Youngner[8] has reported the isolation of heat-stable variants of several types of poliovirus. All the variants have the following properties. They have the same thermal stability as the parent strain at 37° C, but are far more stable at 50° C. Close inspection of that author's published data reveals (1) at 37° C the inactivations, which follow first-order kinetics, have essentially one component to the inactivation curves (the intercept of the slow inactivating component approaches 100 per cent); (2) at 50° C the inactivation curve of the parent is always two-component and the inactivation curve of the heat stable variant is sometimes two-component and (3) the rate of inactivation of the slow inactivating component of the parent at 50° C is always the same as that of the second component (or only component) of the heat stable variant at 50° C. Therefore it is seen that the constant b of the above hypothesis (which controls the reaction rate at low temperatures and the reaction rate of the slow-inactivating component at higher temperatures) is the same for both parent and variant. However the extrapolated intercept for the slow inactivating component is higher in the case of the variant. Although the published data are not sufficient to decide the point unequivocally, it is possible that the constant a is not changed from parent to variant, either. If this is true, it means

that the rate of inactivation by both collapse and chain break mechanisms is the same for both parent and heat stable variant. The variant differs from the parent only in the rate at which the two postulated viable states of nucleic acid are interconverted. These considerations predict conclusions confirmed by the data [9] that it should be difficult or impossible to isolate these variants by heating in the low temperature range, and that it should be impossible to isolate further variants from the original stable variants that are appreciably more stable than the original variants. Furthermore, heating beyond a certain time in the high temperature range should not increase the percentage of heat stable variants in the surviving population. It seems that these strange heat stable

fectivity is coincident with the loss of serologic properties. This is known to be the case for poliovirus inactivations.[9] Since antigenic properties are considered to reside chiefly in the protein portions of the virus, one possible ex

hypothesis, postulating nucleic acid inactivation as the primary mechanism

itself unstable at the ...

It seems that the inactivation of poliovirus by formalin may be a special case of the hypothesis presented here. Formalin is known to bind to proteins in ... more reversible than others. This is presumably ... Therefore the usual mecha... ises is that inactivation occurs ... number of critical sites. Apparently, this postulate is necessary to account for the first order kinetics observed for these reactions.

I now present an alternative explanation for the action of formalin in inactivating poliovirus. In this view the irreversible reactions of formalin with the virus would be incidental to virus inactivations. The importance of formalin would lie in the fact that it breaks, probably in a reversible manner, a small percentage of the hydrogen bonds or other linkages that give the virus nucleic acid its secondary structure. This then would reduce slightly both the ... and $\Delta S\ddagger$ controlling the reaction rate constants in such a manner that th... would become more heat sensitive than normal at any given temp...

Consequently, the primary mechanism involved in the formalin inactivation of poliovirus would be a thermal inactivation one

There are several points of comparison between thermal and formalin type inactivations that argue in favor of factors common to both These points, based extensively on the data of Timm and his co-workers [10] are (1), two-component inactivation curves, each component inactivating with first-order kinetics (2) the extrapolated intercept of the second component increases as temperature decreases approaching 100 per cent for temperatures sufficiently low and (3) the $\Delta H\ddagger$ for inactivation of the second component, either with or without formalin is the same within limits of error, at about 30 kcal /mole The $\Delta H\ddagger$ for inactivations of the fast inactivating component either with or without formalin cannot be determined from the small amount of data published It is important to determine this $\Delta H\ddagger$ value for, if it is high and about the same (50 to 100 kcal mole) in both cases it is compatible with the thermal inactivation hypothesis, while difficult to explain on the basis of the reaction of formaldehyde with a single site Another point to notice, although not a point of comparison is that the extrapolated intercept of the second component is a function of formalin concentration As formalin concentration is increased, the extrapolated intercept of the second component decreases

Turning now to other explanations for the inactivation of poliovirus by formalin it is found that each of these is in conflict with at least one of the above mentioned facts (taken from the data of Timm *et al* [10]) First, hypotheses based upon the assumption that the two-component inactivation curves result from an initially nonhomogeneous population (Salk and Gori, elsewhere in this monograph) cannot account for the fact that the extrapolated intercept of the second component is a function of temperature (that is that the percentage of the population that is relatively resistant is not a constant)

A hypothesis due to Gard [9] explains the shape of the formalin inactivation curves on the assumption that formaldehyde reacting at noncritical sites on the virus slows the rate of reaction of formaldehyde with virus critical sites This hypothesis in its mathematical form predicts that if log per cent survival is plotted against $F \times t$ (where F is formalin concentration and t is time) rather than against t alone, all inactivation curves obtained at different formalin concentrations (other factors remaining constant) will coincide This is the case because the formalin concentration appears only as a constant multiplier of the time variable in all exponential terms of the expression Therefore F is a scaling factor on the time axis As mentioned previously the data of Timm *et al* [10] show that the extrapolated intercept of the second component is a function of formalin concentration which means that such curves cannot be superimposed merely by using a scaling factor on the time axis

Admittedly there is little evidence other than curve shape analogy to favor the view that formalin inactivations are primarily thermal inactivations I believe however that the hypothesis could be reinforced or disproved by accurate kinetic studies as a function of temperature and formalin concentration on both the virus and the infective free RNA Possibly it will be necessary to determine other parameters for example, $\Delta H\ddagger$, pH dependence and ionic strength dependence in order to decide this point It is possible however, that

at the rate of inactivation by both collapse and chain break mechanisms is the same for both parent and heat stable variant. The *variant differs from the* parent only in the rate at which the two postulated viable states of nucleic acid are interconverted. These considerations predict conclusions confirmed by the data [9] that it should be difficult or impossible to isolate these variants by heating in the low temperature range, and that it should be impossible to isolate further variants from the original stable variants that are appreciably more stable than the original variants. Furthermore, heating beyond a certain time in the high temperature range should not increase the percentage of heat stable variants in the surviving population. It seems that these strange heat stable

...on of viruses is that in the high temperature, high $\Delta H\ddagger$ range the loss of infectivity is coincident with the loss of serologic properties. This is known to be *the case for poliovirus inactivations* [9] *Since antigenic properties are considered to reside chiefly in the protein portions of the virus one possible explanation of these facts is that protein denaturation causes the loss of virus infectivity* in the high temperature range. However, it is easy to account for the loss of antigenic properties coincident with infectivity by means of the above hypothesis postulating nucleic acid inactivation as the primary mechanism. The argument is as follows: it is known that DNA stabilizes albumin against thermal denaturation.[13] This fact implies that nucleic acid of the virus stabil...

...self unstable at those temperatures.

It seems that the inactivation of poliovirus by formalin may be a special case of the hypothesis presented here. Formalin is known to bind to proteins in many ways, some of which are more reversible than others. This is presumably ... nucleic acid.[17] *Therefore, the usual mecha*... viruses is that inactivation occurs ... f a number of critical sites. Apparently this postulate is necessary to account for the first-order kinetics observed for these reactions.

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...nd its secondary structure. This then would ... and $\Delta S\ddagger$ controlling the reaction rate constants in such a manner that the virus would become more heat sensitive than normal at any given temperature.

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a simple way of deciding this point may be to study the inactivation kinetics at different temperatures of a given strain of poliovirus and one of its heat stable variants in various concentrations of formalin If the formalin inactivation were primarily a heat inactivation, the same inactivation kinetics for the two viruses could be expected at one temperature, but the stable variant should be more resistant to formalin type inactivation at a higher temperature

One prediction of this hypothesis is that it should be possible to make satis factory vaccines by thermal inactivation alone, if inactivations are carried out at sufficiently low temperatures (that is, temperature ranges where the postu lated collapse mechanism of thermal inactivation is inoperative)

In conclusion, I have attempted to develop a hypothesis that unifies most, if not all of the phenomena associated with animal virus thermal inactivations It is hoped that the hypothesis will be useful in understanding virus structure and function and in explaining other thermal phenomena in biological systems The hypothesis involves the postulate that thermal damage to virus nucleic acid is the primary factor in the process Furthermore, it is necessary to postu late that virus nucleic acid is capable of existing in two viable states and that inactivation can occur by two mechanisms, one involving collapse of secondary structt ... n the nucleic acid ch ... either ... For example, it makes specific predictions concerning the effect of ... heating or of preheating at one temperature, before thermal inactivations oc cur at other temperatures Perhaps the most intriguing outcome of the above hypothesis is that it predicts that nucleic acid may exist in two viable forms It is possible that the difference between the two forms is trivial However the difference is possibly of fundamental biological importance

Acknowledgments

... ons in the prepa ra ... mulating discus sio ... for many useful suggestions and criticisms, Frank Black, for kind help in collecting material, and Candace Vogler, for timely assistance in preparation of the manuscript

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multiplicity reactivation remains the most suitable explanation for the phenomenon described.

The interfering activity of inactivated virus in tests for surviving virus is well established.[4,5] A large dose of UV- or heat treated virus may not yield evidence of HA production in chick embryos, whereas a dilute inoculum of the same preparation may do so. This paradoxical result is explained on the basis that the inactivated virus particles induce interference, so that spread of the infection initiated by the surviving fraction is largely prevented. Upon serial dilution of such seeds, a point is reached at which the available inactivated virus particles no longer suffice to cause extensive interference, and hemagglutinin production becomes measurable. If, however, the amount of surviving virus cannot be diluted beyond the minimal concentration of inactivated virus necessary for this degree of interference, its detection offers greater difficulties. It has been shown that the establishment of solid interference requires considerable time.[6,7] Thus, if a mixture of inactivated infectious virus is injected into chick embryos, the first infectious cycle may proceed undisturbed, but second and further cycles are largely or totally prevented. It was assumed generally that the first-cycle infectious progeny could be detected readily by subinoculation into fresh chick embryos, any residual inactive virus from the primary inoculum being diluted out sufficiently in the process of passage. This assumption is now very much in doubt since it was shown by Isaacs and his associates[8,9] that, upon adsorption of inactivated virus, cells may release a substance referred to as interferon. This substance, devoid of any specific viral HA or complement fixing activity, is capable of inducing resistance to viral infections in susceptible cells. It may be expected, therefore, that interferon produced in the cell systems used for assay of surviving virus may prevent ready detection of infectious virus on subpassage.

The demonstration of multiplicity reactivation, interference, and interferon production seems to depend, to a considerable extent, upon the methods employed for viral inactivation. Accordingly, the phenomena discussed apply to a limited number of inactivation procedures. They must be kept in mind, however, when the nature of the process of inactivation is obscure.

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I. W. McLean, Jr. (*Parke, Davis & Company*): In this monograph repeated reference is made to two phenomena associated with the kinetics of the poliovirus-formaldehyde reaction originally observed in my laboratory. Three years ago, in a series of papers presented at the Federated Meetings in Atlantic City, N. J., the apparent rapid initial inactivation of poliovirus infectivity dur-

Part IV. Problems to be Considered in the Inactivation of Viruses

PANEL DISCUSSION

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WERNER HENLE (*University of Pennsylvania, Philadelphia, Pa*) In this monograph much is said concerning inactivation of viruses by various means but relatively little attention is paid to problems encountered in assays for surviving virus In the determination of rates of inactivation where the samples taken for study still contain appreciable amounts of infectious virus, fewer difficulties are experienced than in tests designed to establish complete loss of infectivity In the former type of study the phenomenon of multiplicity reactivation which conceivably could cause changes in the slopes of inactivation curves, must be considered In the latter type of analysis interference of the inactive virus with the propagation of the surviving fraction offers a well known pitfall among others These points can be documented by experimental observations made with influenza viruses

Multiplicity reactivation has been demonstrated with influenza virus preparations that were partially inactivated by ultraviolet (UV) irradiation[1] In kinetic experiments in chick embryos using as seeds (1) irradiated virus with a ratio of inactive to infectious virus of 10^3, and (2) a 10^{-3} dilution of a nonirradiated portion of the viral suspension (both inocula thus contained similar concentrations of infectious virus), the resulting growth curves, contrary to expectation, were not alike The hemagglutinins (HA) reached detectable and thereafter maximal levels earlier, and the infectivity (ID_{50}) titers were more than tenfold higher in the series injected with UV treated virus than in that inoculated with untreated seed The irradiated inoculum behaved, in fact as if it contained between 10 and 50 times the amount of infectious virus actually measured as present Upon dilution of the irradiated seed or upon more extensive inactivation of the virus this effect was lost These results corresponded in every aspect to those obtained in multiplicity reactivation of irradiated bacteriophage[2] The interpretation of the influenza data on this basis was questioned however by Cairns,[3] who proposed the following alternate explanation The presence of large quantities of inactive virus in the inoculum leads to destruction of cell receptors, so that any viral progeny liberated no longer can be adsorbed onto remaining susceptible cells This results in a more rapid accumulation of free virus in the allantoic fluid than becomes evident after inoculation of the same dose of infectious virus in the absence of inactive virus While this interpretation could and possibly in part, does explain the results obtained in allantoic fluid titrations, it fails to account for the data obtained with the allantoic membrane suspensions or cell associated virus Prevention of adsorption of progeny should if anything lower the HA and ID_{50} titers in the tissue suspensions In fact levels 10 to 100 times higher were obtained in the first infectious cycle in the series of chick embryos injected with irradiated virus than in that inoculated with the appropriately diluted native seed Thus

of the duration of the lag phase to the extent of formalin treatment. With prolonged periods of 1:4000 formalin treatment (up to 16 days at 37° C.), detection of residual infectivity has been delayed for up to 30 days in routine tissue culture safety tests of the formalinized poliovirus vaccine. In my experience the lag or delay has been induced only by treatment with formalin; other chemical and physical methods of inactivation studied have shown no increase in the lag phase. The finding is not related to interference by an excess of inactive poliovirus particles, since it does not occur in virus preparations inactivated by other means. Also, studies in which trace amounts of untreated poliovirus are added to dialyzed or neutralized formalin treated poliovirus vaccine have ruled out the possibility that concentrations of formalin killed virus present in the tests interfere with the initiation of infection by added untreated active virus particles. On the basis of these studies, it is concluded that the active particles remaining following formalin treatment differ intrinsically from untreated virus by being slower in initiating cell degeneration in tissue cultures.

Further studies have been directed toward understanding how these residual virus units differ from the bulk of the virus population. Possible selection through formalin treatment of a slow growing and possibly attenuated variant of the poliovirus has been considered. However, virus clones originating from early and late plaques showed no difference in formalin sensitivity, rate of growth, or ability to initiate infection and degenerative changes in susceptible cells with release of virus. Furthermore, by measuring rate of increase in plaque size, it was shown that plaques appearing after 6 days developed in size at the same rate as plaques appearing after the normal interval of 2 to 3 days. Thus, once infection is initiated by the formalin-damaged virus, its progeny can multiply and infect new cells in a normal manner. It was found that if the buffer capacity of the overlay is increased by the addition of serum and additional sodium bicarbonate, the lag phase could be decreased but that the incubation period required for detection of the control plaques also was decreased so that a relative difference was maintained. This observation confirmed the effect of increasing pH on the rapidity with which residual virus can be detected in tissue culture safety tests, a finding originally reported by Hampil and her co-workers.[8]

The presence of viral aggregates or viral particles occluded by cell debris in poliovirus preparations has been incriminated in certain instances of failure to inactivate. Such occluded and protected virus particles might not be able to infect cells until unmasked by enzymatic action or some other mechanism and thereby might be slow in initiating infection of cells. To investigate this possibility, aliquots of a lot of Mahoney strain virus were inactivated partially by treatment with formalin or ultraviolet light as indicated (TABLE 1), and one part was retained untreated as a control. These preparations were passed serially through matched S and S-membrane filters* of decreasing pore size, and samples were removed for testing after each filtration. The results given in TABLE 1 show clearly that there is no change in the percentage of plaques developing late with the formalinized preparation, even after the presence of any large aggregates has been excluded by passage through a membrane of al

* Obtained from Schleicher & Schuell Co., Keene, N. H.

ing the first few hours of formalin action was described as well as the so-called delay phenomenon related to decreased ability of formalin treated poliovirus to initiate infection of cells Since these effects may well be different aspects of the same mechanism it should be pertinent to review the reported findings[1-4] and present additional data given last year at the Seventh International Congress for Microbiology held in Stockholm Sweden[4]

My colleagues and I have shown[1] that the rapid initial drop in titer is not eliminated by varying many of the conditions of the reaction or the virus assay procedure Among the inactivation conditions studied were pH over the range of 6 5 to 8 0, formalin concentrations from 1 2000 to 1 8000 the presence of increased and decreased concentrations of amino acids and protein the degree of filtration, the effect of sonic vibration the effect of neutralizing the formaldehyde and testing of samples in tissue culture immediately, or after storage under various conditions in the cold for up to 12 weeks It was reported[4] that the early drop was triggered by having the sample at 37° C when the formalin is added even if it is immediately chilled or neutralized but unless the sample was warmed the drop did not occur The method of assay of residual activity *in the samples did not eliminate the effect* *Even prolonged holding of the* original test cultures or blind subcultures (total time of observation 28 days) did not change the general shape of the curve, even though the slope or apparent inactivation rate was decreased The rapid initial drop is less evident however, when the color change or metabolic inhibition (MIT)[5] method of assay is employed than when the Dulbecco plaque technique[6] or development of CPE in stationary or roller tubes[7] and plastic panels[8] is used as the assay procedure Also without changing the result, a variety of cell types of varying sensitivity to poliovirus has been utilized for assay of viral activity

The delay phenomenon also has been studied extensively and my co-workers and I have reported[2,3] that it can be demonstrated in agitated or stationary tube or bottle cultures utilizing a number of different tissue culture cells, and is of special importance in tissue culture safety testing of formalinized poliovirus vaccines It should be noted that the MIT method of assay tends to mask the delay phenomenon since acid frequently will develop in the cultures before the formalinized virus initiates cell infection The plaque technique however has been very useful in these studies, since it allows a clear estimate of the lag phase for each infectious unit remaining All three types of poliovirus show the delay effect but it is usually more evident with Type I strain and less marked in the first 8 to 10 hours of formalin treatment of Type III My co-workers and I also have reported[3] similar results with other viruses treated with *formaldehyde adenovirus vaccinia* Eastern and Western equine and certain Echo and Coxsackie types It is evident that this phenomenon must be considered in evaluating the safety of any virus vaccine prepared by formalin treatment The degree of delay is correlated directly with the decrease in titer of the formalin treated virus and therefore, with the length of exposure to and concentration of formaldehyde However, qualitatively neither the formalin concentration or variation of other inactivation conditions changed the result Also neither initial virus titer nor partial previous inactivation by heat or ultraviolet irradiation nor degree of pretreatment filtration affect the relationship

the virus titer and rate of plaque appearance. With both the ultraviolet-treated virus and the control, no significant change was observed as a result of the washing. However, the formalin treated preparation showed a decrease both in titer and in the percentage of delayed plaques produced. Several interpretations are possible, but it seems most probable that the formalin-dam

TABLE 3
EFFECT OF STORAGE OF SAMPLES ON INFECTIVE TITER AND PLAQUE APPEARANCE TIME

Test day	Cont.	(No HCHO)	Stored*	HCHO neut	+	Stored*	Dialyzed	+	Stored*
	1 day	1 wk	12 wks	1 day	1 wk	12 wks	1 day	1 wk	12 wks
3	66	62	72	0	0	35	0	2	26
4	97	90	100	14	19	71	0	17	41
5	100	100	—	14	54	89	36	69	69
6	—	—	—	88	94	97	50	95	81
7	—	—	—	100	100	100	79	100	83
Pfu/ml ($\log_{10}$)	8.0	8.3	7.9	1.2	2.2	2.4	1.5	2.2	2.4

* Poliovirus Type 1 (Mahoney) formalin 1:2000 16 hours at 37° C pH 7.0. Timed from completion of treatment.

TABLE 4
EFFECT OF NEUTRALIZING WITH DAMDONE AND STORAGE ON FORMALIN TREATED POLIOVIRUS TYPE 1 (MAHONEY STRAIN)

Initial treatment		Preformalin 37° C	Time at 37° C with formalin (1:4000) 0 hours	¼ hour	1 hour	2 hours	4 hours	8 hours	16 hours
Neutralized	Pfu/ml $\log_{10}$*	8.20	8.03	8.11	7.73	7.53	6.98	5.75	4.03
	3-7 day plaques (%)	85	72	77	81	64	52	47	5
Not neutralized	Pfu/ml $\log_{10}$*	8.35	7.18	6.66	6.82	6.60	6.14	5.43	3.95
	3-7 day plaques (%)	92	48	29	30	11	30	25	1

* $\log_{10}$ pfu/ml.
All samples immediately diluted 1:100 in Medium 199 and stored at 4 to 6° C for 7 days before testing.

aged virus is less able to adsorb to the cell surface and also is dissociated readily after adsorption but before actual infection has occurred.

In a number of experiments we have seen apparent recovery of virus activity and decrease in the delay effect following storage of bisulfate neutralized or dialyzed samples. Results of one such experiment are shown in TABLE 3. The cumulative percentages of the total number of plaques appearing during 9 days of observation of the cultures are listed for the third to seventh days of the test. Note the continuing recovery of the ability to induce early plaque formation during prolonged storage of the treated samples before testing, even though the greatest recovery in titer occurred during the first week.

Results of another experiment bearing on this problem are given in TABLE 4

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untreated
tion or most probably, no
delay effect is not caused by aggregation or occlu ion of virus

TABLE 1

EFFECT OF SEQUENTIAL FILTRATION ON INFECTIVE TITER AND TIME OF PLAQUE APPEARANCE FOR POLIOVIRUS TYPE 1 (MAHONEY)

Sample treated		Treated and unfiltered	Membrane filtered range of pore sizes (mμ)					
			750 to 1000	500 to 750	200 to 500	80 to 200	50 to 80	10 to 50
Control untreated fluid	Pfu/ml log 10 3/7 day plaques (%)	7 8 78	7 7 82	7 7 83	7 6 86	7 5 87	3 6 94	Neg —
Formalin 1 4000 hours 37° C pH 7 0	Pfu/ml log 10 3/7 day plaques (%)	4 1 42	4 1 40	3 8 44	3 0 36	1 7 39	Neg —	Neg —
Irradiated UV 18 watts 600 ml / min	Pfu/ml log 10 3/7 day plaques (%)	3 7 81	3 5 81	3 6 92	2 7 89	1 7 100	Neg —	Neg —

TABLE 2

EFFECT OF REMOVAL OF UNADSORBED VIRUS ON RATE OF PLAQUE APPEARANCE

Type of poliovirus	Treatment after adsorption for 2 hours at 37 C	1 4000 Formalin 24 hours 37 C dialyzed		Ultraviolet 18 watts 600 ml./min		Control untreated	
		Log 10 pfu/ml.	Percentage plaques day 3	Log 10 pfu/ml.	Percentage plaques day 3	Log 10 pfu/ml.	Percentage plaques day 3
I Mahoney	None Washed	1 8 0 4	3 20	3 9 3 7	94 97	8 2 7 9	84 93
II MEF 1	None Washed	3 2 2 7	18 57	2 7 2 4	73 89	7 3 7 1	80 85
III Saukett	None Washed	3 2 2 8	81 82	2 7 2 4	85 87	7 3 7 1	91 94

The experiment summarized in TABLE 2 was performed to determine whether the delay effect was due to a decrease in the ability of the virus to adsorb to the cell or to the inability to infect or initiate multiplication following adsorption The three types of poliovirus showed qualitatively similar results The virus preparations were allowed to remain on the monkey kidney cell sheets for 2 hours at 37° C in order that adsorption of virus to the cells could take place, then one half of the cultures was washed 3 times with media to remove any unadsorbed virus the remaining bottles were retained unwashed as controls Both preparations were overlaid with agar and incubated to determine

diffusing into the center of the poliovirus to affect the nucleoprotein Only after the secondary reaction with the nucleoprotein is complete can the virus be termed completely inactivated In the late samples a further decrease in [illegible]

occur in tests of these concentrated virus samples cannot be ruled out as a contributing factor in the tailing observed I accordingly believe that the formal-

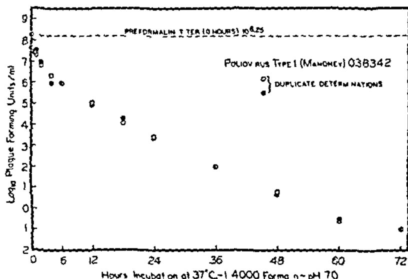


FIGURE 1 Formalin inactivation of poliovirus arithmetic time plot

dehyde poliovirus inactivation curve can be described best as the summation of three functions, each of which may predominate depending upon the inactivation conditions treatment and storage of the samples, and type of assay procedure used for determining the amount of residual virus Perhaps Woese can supply the unifying mathematical concept, but for the moment we are content to recognize that the reaction is complex and oversimplification hazardous

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In this experiment a Seitz filtered preparation of Mahoney strain virus was treated with 1:4000 formalin at pH 7 and 37° C, while samples were removed at doubling time intervals up to 16 hours. Each sample was chilled immediately, divided into two equal parts, and one half treated with equimolar Damidone,* a highly efficient formalin binding agent. Each sample was diluted 1:100 and stored for 7 days at 4° to 6° C before testing. The Damidone treated samples show less delay and a higher titer than do the untreated samples, but the difference becomes less marked as the time of formalin treatment is extended, so that after 16 hours the neutralized and nonneutralized samples were not significantly different in titer or degree of plaque delay. A concentration of 1:400,000 formalin, equivalent to the 1:100 diluted samples when added to another sample of the same poliovirus preparation at refrigerator temperature did not reduce the titer significantly or increase the lag phase in tests after storage under the same conditions for 1 week. The relative reduction in titer and delay observed with the nonneutralized samples must, therefore, be a result of combination of the formaldehyde with the virus during the period at 37° C before chilling and diluting. Either the inactivation process continues in the cold or during the assay procedure, or the formation of the initial complex is reversed when the formaldehyde is neutralized by Damidone. If the latter explanation is correct, reversal apparently becomes more difficult as the formalin treatment at 37° C is extended.

In conclusion, FIGURE 1 presents data that are typical of the results obtained consistently in our formalin inactivation rate studies. Space does not permit a detailed description

uniform manner and the

ing the virus and washing,

gelatin technique that allows quantitative recovery of the virus acivity[18]. Two samples were prepared and tested for each point, and the final virus pellets were concentrated 10 or 100 times for the tests on the longer treatment times. It is extremely important in studying the kinetics of biological systems to attempt to cover the full reaction with a large number of points; this study was an attempt to obtain a complete and quantitative picture of the reaction with a minimum of extraneous effects from treatment of the samples, assay variation, and reactivation. No lines have been drawn because I do not feel that any single reaction describes the data. Rather, there are three zones

Thus, this surface effect of the formaldehyde on the

in both the phenomena discussed. With time these surface changes become more extensive and less reversible while, simultaneously, the formaldehyde is

* Obtained from Eastman Kodak Co., Rochester, N. Y.

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